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Page	Line	For	Read
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,,	31	k_M	k_M
821	12	524 m	524 mμ
,,	FIG. 5	$1\times10^{3}\ M$, $1\times10^{2}\ M$	$1\times10^{-3}\ M$, $1\times10^{-2}\ M$
31	FIG. 3	4-Deoxypyridoxine,	4-Deoxypyridoxine phospha
52	9	100 moles	100 μmoles
56	Table III (column 2)	10^{-2}	10^{-3}
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58	Table IV (column 4)	from reduced nile blue	from DPNH	from reduced nile blue	from DPNH
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The Journal of Biochemistry, Vol. 47, No. 1, 1960

THE INCORPORATION OF LABELLED PHOSPHATE INTO THE PHOSPHOLIPIDS OF THE AVIAN TUBERCLE BACILLI*

By YOSHIO TANAKA

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(*With the technical assistance of* HIDEO TANNO AND HIROSHI SUZUKI)

(Received for publication, October 8, 1958)

The biosynthesis of phospholipids in animal tissues has been studied by many investigators (*1–14*). However, there is as yet little information on this subject in microorganisms (*1*). From the point of view that the enzymes concerned with the metabolism of phospholipid may play important roles in the physiology, especially in the pathogenity (*15*), of the tubercle bacilli, studies on the mechanism of phospholipid synthesis by the avian tubercle bacilli were performed with reference to Dawson's investigations (*2*, *4*, *16*) on the same subject in animal tissues.

The present paper deals with the incorporation of the labelled phosphate into the phospholipids of avian tubercle bacilli under various conditions.

METHODS

Preparation of Bacterial Suspensions—Avian tubercle bacilli (strain *Takeo*) were cultivated on modified Long's synthetic liquid media (sodium glutamate was used in place of asparagine) for 5 days. The harvested bacilli were washed twice with 0.85 per cent NaCl solution followed by 0.1 *M* veronal buffer, pH 7.0. In general, the washed bacteria (900 mg.) were suspended in 3 ml. of the incubation medium.

Composition of the Incubation Medium—Veronal buffer, pH 7.0, 0.1 *M*; $MgCl_2$, 0.017 *M*; pyruvate, 0.03 *M*; fumarate, 0.0033 *M*; NaF, 0.017 *M*; glycerol, 0.008 *M*; P^{32} (ortho phosphate), 10 μc. per ml. of the incubation medium. The medium was prepared with reference to Dawson's report (*2*).

Methods of Incubation—(A) The washed cells were suspended with or without inhibitor in the incubation medium. (B) The washed cells were shaken with or without inhibitor in 0.1 *M* veronal buffer, pH 7.0 (300 mg. wet weight of cells per ml. of the buffer) for 4 hours at 37° and then washed twice with 0.1 *M* veronal buffer before the incubation. (C) The washed cells were shaken with or without inhibitor in 0.125 *M* veronal buffer, pH 7.0 (300 mg. of cells per 0.8 ml. of buffer) for 4 hours at 37° and a concentrated incubation medium was added to the cell suspension to make the composition of the incubation medium same as that in the methods A and B. All the tests were carried out at 37° for one hour.

* A part of this work was delivered at the third Symposium on Metabolism at Sendai, January 15, 1957, and the outline was presented at the 30th Annual Meeting of the Japanese Biochemical Society at Kyoto, July 16, 1957.

Extraction of Phospholipids from Bacteria—At the end of the incubation period, 3 ml. of 20 per cent (*w/v*) trichloroacetic acid solution was added to the incubated suspension. The mixture was centrifuged at $1{,}000 \times g$, and the precipitate was washed with 5 ml. of 5 per cent trichloroacetic acid solution and three times with 5 ml. portions of water, and extracted with 10 ml. of a mixture of methanol and chloroform (1 : 1) for one hour at 80° under a reflux condenser. The extract was washed with 10 ml. of 0.25 *M* $MgCl_2$ solution in a separating funnel. One ml. of the $CHCl_3$ layer (phospolipid fraction was transferred to a dish and dried for the determination of radioactivity. Another 1 ml. of the $CHCl_3$ layer was removed for the determination of phosphorus content. The centrifuged extraction residue (residual fraction) was washed with the methanol-chloroform mixture and suspended in methanol, then the phosphorus content as well as radioactivity was determined.

Fractionation of Phospholipids—Ten ml. of the phospholipid extract was fractionated according to the method of Dawson (*16*) (Scheme 1). The four fractions obtained by

SCHEME 1

The Fractionation Procedure of Phospholipid

Phospholipid extract

the extract was taken to dryness; the residue was dissolved in 10 ml. of boiling methanol and cooled for 30 minutes at 0°.

→ Ppt. of methanol-insoluble fraction was washed twice with 5 ml. of methanol.

the solution was filtered through glass wool and treated with 200 mg. of MgO.

→ MgO-adsorbed fraction was washed with 3 ml. of methanol.

→ Acid phospholipid (Cephalin)

the solutions were taken to dryness and the residue was incubated for 20 hours at 37° with 3 ml. of 1 *N* NaOH, then the solution was cooled in ice and 3 ml. of 1 *N* HCl and 4 ml. of 20% TCA were added.

→ Saponified fraction Lecithin)

→ Unsaponified fraction (Sphingomyelin) was washed with 10% TCA on the filter paper.

this procedure were as follows: methanol-insoluble fraction (acid phospholipids, 'cephalin' fraction), MgO-adsorbed fraction (acid phospholipids, 'cephalin' fraction), MgO-unadsorbed saponified fraction (lecithin fraction), MgO-unadsorbed unsaponified fraction (sphingomyelin fraction).

Paper Chromatography of Phospholipid Subjected to Hydrolysis—Phospholipid extract was taken to dryness in vacuo at room temperature. The phospholipid residue was then taken up in 0.8 ml. of carbon tetrachloride and to the solution was added 6.3 ml. of methanol and 0.9 ml. of water. The mixture was then treated with 2.0 ml. of 1 *M* methanolic NaOH and immediately incubated at 37° for 15 minutes by the method of

Dawson (*4*). After cooling in ice the hydrolysate was diluted with 16 ml. of ice-cold water and passed through a column of Amberlite IRC 50 resin. The ice-cold eluate was shaken successively with 25 ml. portions of carbon tetrachloride, ether, petroleum ether and iso-butyl alcohol. The resulting aqueous layer was filtered, neutralized to pH 7.0 with ammonia and taken to dryness under reduced pressure below 50°. The residue was taken up in a little water and subjected to two-dimensional paper chromatography. The filter papers used were well washed with 2*N* acetic acid prior to chromatography. Phenol/NH_3 solvent (phenol saturated with 0.1 per cent (*w/v*) NH_3 solution) and butanol/trichloroacetic acid solvent (a mixture of tert-butanol and water (62 : 38) (*v/v*) containing 10 per cent (*w/v*) trichloroacetic acid) were used for descending and ascending chromatography, respectively. The chromatogram was dried in air and trichloroacetic acid was removed by washing the chromatogram in ether. The spots were located by spraying with ninhydrin and then with acid-molybdate reagent of Hanes and Isherwood (*17*).

The determination of phosphorus was carried out by the method of Berenblum and Chain (*18*). The optical density was measured in a spectrophotometer at 700 mμ.

Radioactivity was measured with a Geiger-Müller counter. Specific acitivity was expressed as counts per minute per mg. of phosphorus.

Oxygen uptake was measured by Warburg's manometric technique at 37° for one hour. The main compartment contained 0.1 ml. of the bacterial suspension and 0.9 ml. of the incubation medium. The center well contained 0.2 ml. of 20 per cent KOH and a small strip of filter paper. Succinic dehydrogenase activity was measured by Thunberg's method using methylene blue (*19*) and its activity was expressed in terms of Q_{MB} according to the method of Corran, Dewan and Gordon (*20*),

$$Q_{MB} = \frac{17}{70} \times \frac{3600}{xy} \times 10^4$$

where x is the time in minutes needed for the disappearance of methylene blue colour and y is the wet weight of bacilli.

RESULTS

Effect of pH—The optimum pH for the incorporation of P^{32} into phospholipid was measured in 0.1 *M* veronal buffers. It is seen from Fig. 1 that the pH optimum is near 7.0–7.5.

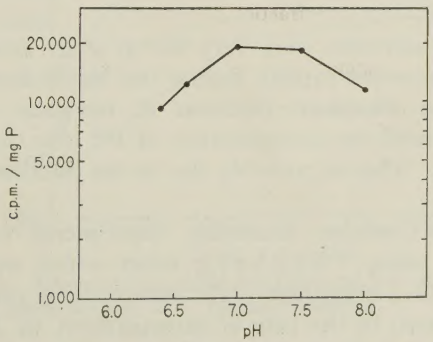

FIG. 1. Effect of pH on the incorporation of P^{32} into phospholipid. Incubation : 1 hour, at 37°.

Effect of Incubation Time at pH 7—Specific radioactivities of the phospholipid increased rapidly during the initial 60 minutes and then very slowly. In the residual fraction, the specific activities increased rapidly until 120 minutes after the beginning of incubation, then decreased slowly (Fig. 2).

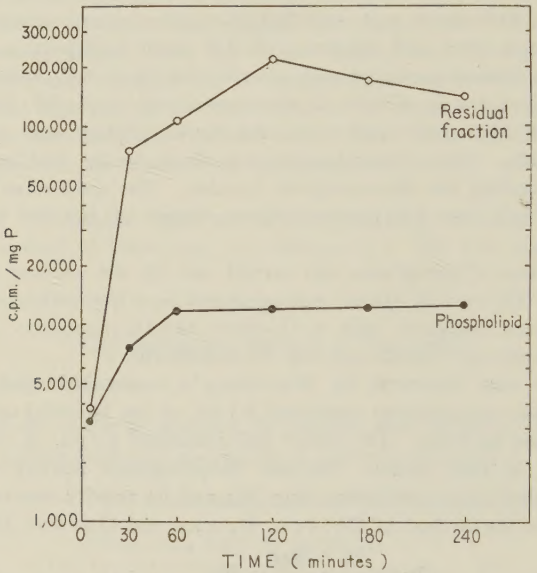

FIG. 2. Effect of incubation time on the incorporation of P^{32}. Incubation: pH 7.0, at 37°.

Relatively high specific activity was observed at zero minutes in the phospholipid and the residual fractions. However, the zero minute value shown in Fig. 2 is not strictly accurate as it takes some minutes for pipetting, homogenation, or other procedures. Therefore experiments were carried out to obtain the specific radioactivities at strict zero minute. Three ml. of 20 per cent trichloroacetic acid solution was added to the bacilli before the incubation medium. After that, the same procedures as those described above were followed.

The specific radioactivities were very low at strict zero minute as shown in Fig. 3, but they increased rapidly during the initial 5 minutes.

Effect of Inorganic Phosphate—Addition of inorganic phosphate to the incubation media reduced the incorporation of P^{32} into the phospholipid and the residual fractions. This is probably due to the dilution effect of inorganic phosphate (Fig. 3).

Effect of Anaerobic Condition—Anaerobic experiments were carried out in nitrogen atmosphere using Thunberg tubes which were designed in our laboratory to be used in a centrifuge (*21*). Anaerobiosis caused a considerable decrease (60–80 per cent) in the rate of incorporation of P^{32} into the phospholipid and the residual fractionations (Fig. 4). If nitrate is utilized by the bacilli as the final hydrogen acceptor, the decrease of the incorporation of

P^{32} into phospholipid by anaerobiosis will be recovered by the addition of

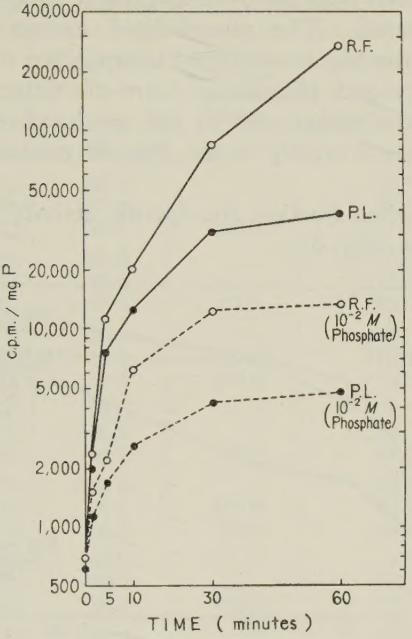

FIG. 3. Decreasing effect of inorganic phosphate on the incorporation of P^{32}. P.L. phospholipid; R.F. residual fraction.

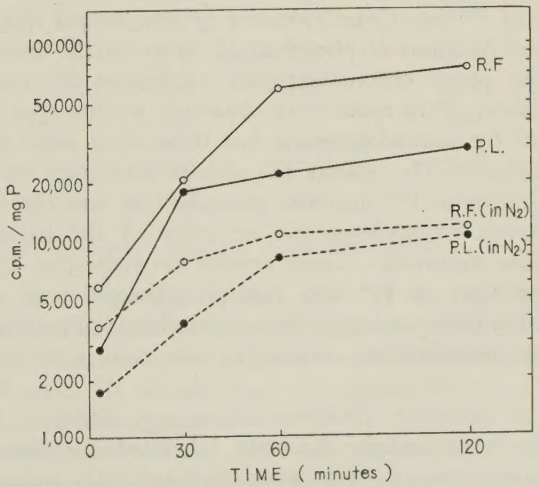

FIG. 4. Effect of anaerobic condition on the incorporation of P^{32}. P.L. phospholipid; R.F. residual fraction.

$NaNO_3$. However, the addition of 0.01 *M* $NaNO_3$ to the system had no effect.

Incorporation of P^{32} into Individual Fraction of Phospholipids—In this experiment 3 g. of the washed bacteria were suspended in 10 ml. of the incubation medium and incubated. The phospholipid extract was fractionated as described before. In the MgO-unadsorbed unsaponified fraction (sphingomyelin fraction), radioactivity and phosphorus were not detectable. In the MgO-unadsorbed saponified fraction and in the methanol-insoluble fraction, the specific activity increased rapidly in the first 30 minutes but the subsequent increase was slow.

In the MgO-adsorbed fraction, the specific activity was lower than those of the other fractions. (Fig. 5).

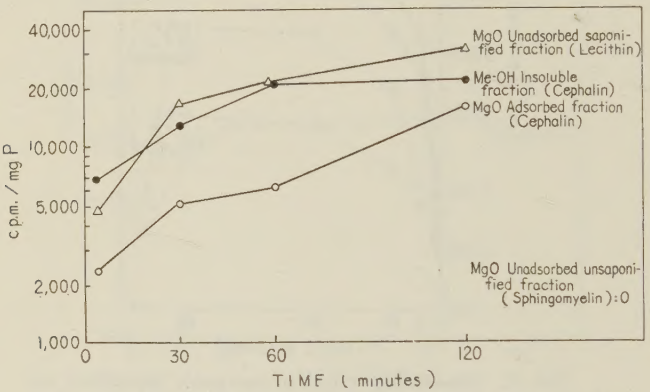

FIG. 5. Incorporation of P^{32} into the individual fraction of the phospholipid.

Incorporation of P^{32} into Certain Fraction of Phospholipid Subjected to Hydrolysis—To study the fractions of phospholipid into which the incorporation of P^{32} occurred, the paper chromatographic technique (*4*) was applied to the hydrolyzed products. Five spots were detected by the spraying method and many other spots by autoradiography, but these spots were not identified.

Effect of Inhibitors—The effects of various inhibitors on the respiration and the incorporation of P^{32} into the phospholipid and the residual fractions were tested. Succinic dehydrogenase activities of the bacilli before the incubation were also measured. These results are shown in Table I.

The incorporation of P^{32} into the phospholipid and residual fractions were more sensitive than respiration to such inhibitors of respiration as cyanide, malonate and mono-iodoacetate, except in one case with malonate used in method B.

Inhibitors of oxidative phosphorylation, *e.g.* arsenate, 2,4-dinitrophenol (DNP) and azide were tested. Arsenate inhibited the incorporation of P^{32} into the phospholipid in all cases and inhibited the respiration slightly in method B. Azide, at a concentration of 10^{-2} *M*, also inhibited markedly the incorporation of P^{32} in both methods A and C. DNP at concentrations of 10^{-2} *M* and 10^{-3} *M* inhibited the incorporation of P^{32} in all cases. When the bacilli were treated with 10^{-2} *M* DNP, mono-iodoacetate, malonate or azide

TABLE I

Effect of Inhibitors on the Incorporation of P^{32}

Inhibitor and incubation method		O_2 uptake	Succinic dehydrogenase activity	Incorporation rate of P^{32}	
				into phospholipid	into residual fraction
Control		100 (%)	100 (%)	100 (%)	100 (%)
KCN	10^{-2} *M* (A)	86.0		22.7	
	(B)	103.0		66.9	71.4
	(C)	98.0	104.0	27.5	1.5
Malonate	10^{-2} *M* (A)	87.0		12.2	14.3
	(B)	138.0		276.0	81.4
	(C)	92.0	47.5	45.0	6.0
	10^{-3} *M* (A)	100.0		101.7	59.2
	(B)	96.4	100.0	116.3	113.7
	(C)	94.3	100.0	55.7	100.0
CH_2ICOOH	10^{-2} *M* (A)	99.0		1.5	8.7
	(B)	86.0		52.5	46.3
	(C)	52.5	43.4	13.4	2.2
	10^{-3} *M* (A)	100.0		45.0	33.8
	(B)	96.4	100.0	53.4	75.3
	(C)	100.0	100.0	45.2	61.2
Arsenate	10^{-2} *M* (A)	96.0		7.9	93.6
	(B)	90.0		61.4	7.5
	(C)	99.0	102.0	29.0	3.9
	10^{-3} *M* (A)	100.3		54.3	27.1
	(B)	96.0	100.0	72.5	75.8
	(C)	101.0	100.0	41.2	43.5
NaN_3	10^{-2} *M* (A)	93.0		1.3	17.0
	(B)	101.0		88.7	109.2
	(C)	106.0	29·2	10.4	5.0
2,4-dinitrophenol	10^{-2} *M* (A)	103.0		1.3	16.9
	(B)	87.0		70.0	69.7
	(C)	10.0	15.7	21.4	1.6
	10^{-2} *M* (A)	101.0		90.0	72.7
	(B)	98.5	100.0	89.2	67.0
	(C)	105.0	100.0	51.0	53.1
Quinine	10^{-2} *M* (C)	15.7	16.1	3.6	18.4

A, B and C in the parenthes show the incubation methods.

for 4 hours in 0.1 *M* veronal buffer, pH 7.0, the succinic dehydrogenase activity and the incorporation of P^{32} were reduced markedly.

The effects of inhibitors were diverse with different concentrations and methods of incubation. It seems that the permeability of the cell may participate in the inhibition process.

Effect of Treatment with Veronal Buffer—In the course of experiments which were designed to test the action of the inhibitors on the incorporation of P^{32} into the bacilli, it was found that treatment of the bacilli for 4 hours at 37° in the buffer without added inhibitor markedly increased the specific activity (Fig. 6). However, the succinic dehydrogenase activity decreased during the treatment in the buffer. The phosphorus contents in the veronal buffer

during the treatment were determined and the results are shown in Table II. The total and organic phosphorus contents in the buffer increased in

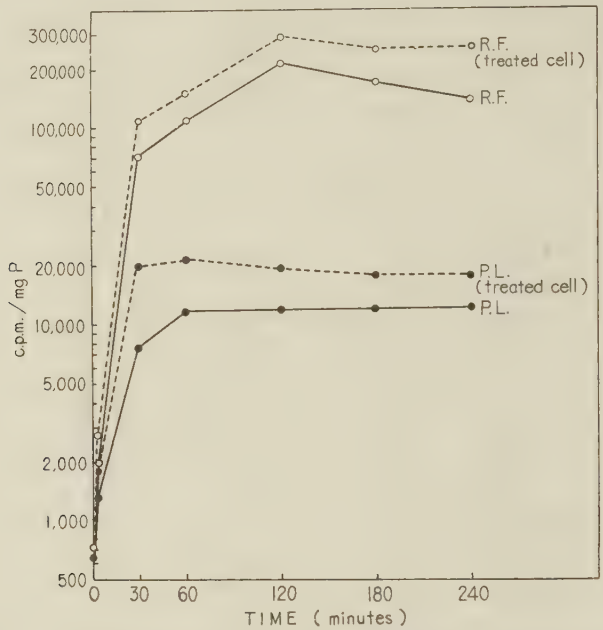

FIG. 6. Effect of treatment in veronal buffer on the incorporation of P^{32}. Bacilli were shaken in veronal buffer pH 7.0, at 37° before incubation. P.L. phospholipid; R.F. residual fraction.

TABLE II

Phosphorus Contents in the Veronal Buffer during Treatment

Shaking time (*hour*)	Total P (*μg./ml.*)	Inorganic P (*μg./ml.*)	Organic P (*μg./ml.*)
0	24.0	15.8	8.2
1	31.9	13.5	18.4
2	28.9	6.3	22.6
4	37.8	4.6	33.2

Bacilli were shaken in veronal buffer pH 7.0, at 37°.
Total P of cell suspension is 5 mg./ml.

proportion to the length of the shaking time, while the inorganic phosphorus content decreased. This result indicates that the phosphorus contents inside the cell decreased. The amount of escaped phosphorus was very small compared to the total phosphorus in the cell, but certain organic phosphorus

compounds important for the synthesis of the phospholipid might be contained in the escaping phosphorus.

Effect of SH-inhibitors—p-Chloromercuri-benzoate (PCMB) and mono-iodoacetate reduced the incorporation of P^{32} into the phospholipid and the respiration, but PCMB (10^{-2} *M*) had no effect in method B. Succinic dehydrogenase, which is an SH-enzyme and is important in the TCA cycle, was reduced by treatment with PCMB or mono-iodoacetate. Metal ions and other reagents which are considered as SH-inhibitors were tested (Table III).

TABLE III

Effect of SH-inhibitors on the Incorporation of P^{32}

Inhibitor and incubation method		O_2 uptake	Succinic dehydrogenase activity	Incorporation rate of P^{32} into phospholipid	Incorporation rate of P^{32} into residual fraction
Control		100 (%)	100 (%)	100 (%)	100 (%)
PCMB	10^{-2} *M* (A)	39.0		24.6	457.2
	(B)	11.0		93.5	100.5
	(C)	10.7	15.7	11.2	1.7
CH_2ICOOH	10^{-2} *M* (A)	99.0		1.4	8.7
	(B)	86.0		52.5	46.3
	(C)	52.5	43.4	13.4	2.2
	10^{-3} *M* (A)	100.0		45.0	33.8
	(B)	96.4	100.0	53.4	75.3
	(C)	100.0	100.0	45.2	61.2
Ferricyanide	10^{-2} *M* (C)	117.0	98.0	81.3	94.4
Cu^{++}	10^{-2} *M* (C)	46.0	16.1	9.1	1.1
Hg^{++}	10^{-2} *M* (C)	24.4	16.1	5.2	1.0
Ag^{+}	10^{-2} *M* (C)	12.6	16.1	5.5	23.0
Fe^{++}	10^{-2} *M* (C)	155.0	96.9	64.2	100.5
P C M B	10^{-2} *M* (C)	58.5	16.1	6.9	1.6
Treated with PCMB (10^{-2} *M*)+cysteine 2×10^{-3} *M*		45.4	16.1	7.2	1.4
P C M B	10^{-3} *M* (C)	68.1	100.0	75.8	91.6
Treated with PCMB (10^{-3} *M*)+cysteine 2×10^{-3} *M*		48.2	100.0	23.8	19.1
P C M B	10^{-4} *M* (C)	69.2	100.0	90.1	91.0
Treated with PCMB (10^{-4} *M*)+cysteine 2×10^{-3} *M*		91.1		86.8	48.6
CH_2ICOOH	10^{-2} *M* (C)	40.4	53.5	12.8	4.9
Treated with CH_2ICOOH (10^{-2} *M*)+cysteine 2×10^{-3} *M*		34.9	53.5	11.6	1.3

A, B and C in the parenthes show the incubation methods.

Cu^{++}, Hg^{++} and Ag^{+} inhibited markedly the incorporation of P^{32}, the respiration, and the succinic dehydrogenase activity; ferricyanide slightly inhibited the incorporation of P^{32} into the bacilli, but the respiration was stimulated by ferricyanide.

Experiments were carried out to see whether there was recovery of the incorporation of P^{32} by the addition of cysteine after the bacilli had been treated with PCMB or mono-iodoacetate. But there was no recovery in the rate of incorporation. These results could be caused by too high a concentration of PCMB (10^{-2} *M*) or mono-iodoacetate (10^{-2} *M*). Experiments were carried out by using lower concentrations of PCMB (10^{-3} *M* or 10^{-4} *M*). In

TABLE IV

Effect of Metal-chelating Agents on the Incorporation of P^{32}

Metal-chelating reagent and incubation method	O_2 uptake	Succinic dehydrogenase activity	Incorporation rate of P^{32} into phospholipid	Incorporation rate of P^{32} into residual fraction
Control	100 (%)	100 (%)	100.0 (%)	100.0 (%)
o-phenanthroline 10^{-2} *M* (C)	96.2	88.5	8.1	30.9
10^{-3} *M* (C)	99.2	100.0	69.0	87.1
Treated with *o*-phenanthroline (10^{-3} *M*)+Fe^{++} 10^{-2} *M*	118.4	100.0	68.1	53.4
Nitroso-R-salt 10^{-2} *M* (C)	117.5	20.6	89.4	131.5
10^{-3} *M* (C)	97.0	111.0	89.0	102.8
Treated with nitroso-R-salt (10^{-3} *M*)+Co^{++} 10^{-2} *M*	103.0	111.0	104.0	104.0
α,α'-Dipyridyl 10^{-2} *M* (C)	88.7	100.0	59.9	87.9
10^{-3} *M* (C)	92.1	111.0	83.5	86.5
Treated with α,α'-Dipyridyl (10^{-3} *M*)+Fe^{++} 10^{-2} *M*	104.0	111.0	26.3	97.1
Oxine 5×10^{-3} *M* (C)	88.2	52.5	89.5	92.2
10^{-3} *M* (C)	81.3	111.0	93.6	100.0
Treated with oxine (10^{-3} *M*) +Zn^{++} 10^{-2} *M*	86.0	111.0	19.0	30.3
NH_2OH 10^{-2} *M* (C)	75.1	23.2	25.2	46.5
10^{-3} *M* (C)	102.1	100.1	100.1	108.0
Treated with NH_2OH (10^{-3} *M*) +I^- 10^{-2} *M*	99.5	100.0	95.5	100.0
EDTA 10^{-2} *M* (C)	101.5	100.0	40.9	55.0
Sodiumdiethyldithiocarbamate 10^{-2} *M* (C)	83.3	91.3	100.0	118.9
10^{-3} *M* (C)	102.2	100.0	100.0	122.9
Fe^{++} 10^{-2} *M* (C)	155.0	96.9	64.2	100.5

C in the parenthes show the incubation method.

these experiments, not only was there no recovery of the incorporation rate but incorporation rate rather decreased by the addition of cysteine.

Effect of Metal-chelating Reagents—Experiments showed that 10^{-2} *M* or 10^{-3} *M* *o*-phenanthroline, 10^{-2} *M* α,α'-dipyridyl, 10^{-2} *M* ethylenediamine tetraacetate (EDTA) and 10^{-2} *M* hydroxylamine inhibited the incorporation of P^{32} into the phospholipid and the residual fractions of the bacilli. The bacilli were

treated with 10^{-3} *M* metal-chelating reagents for 4 hours and then 10^{-2} *M* of the corresponding metal ions (*o*-phenanthroline and α,α'-dipyridyl correspond to Fe^{++}, nitroso-R-salt, oxine and hydroxylamine correspond to Co^{++}, Zn^{++} and I^-, respectively) were added to the incubation media. The P^{32}-incorporation was inhibited by 10^{-3} *M* of *o*-phenanthroline and 10^{-3} *M* of α,α'-dipyridyl, and Fe^{++} (10^{-2} *M*) also did the same action. It is supposed, therefore, that Fe^{++} is necessary for the incorporation of P^{32} into the phospholipid but is inhibitory at a higher concentration. Zn^{++} may be an inhibitor, since 10^{-3} *M* of oxine had no effect on the P^{32}-incorporation and Zn^{++} (10^{-2} *M*) was inhibitory. Nitroso-R-salt (10^{-3} *M*) slightly inhibited the incorporation of P^{32} into the phospholipid but the addition of Co^{++} (10^{-2} *M*) stimulated, indicating Co^{++} being an activator. The addition of hydroxylamine (10^{-3} *M*) and of I^- (10^{-2} *M*) did not affect the P^{32}-incorporation, and I^- probably does not concern in the process (Table IV).

TABLE V

Effects of Cofactors and Some Biochemical Agents on the Incorporation of P^{32}

Cofactor and incubation method		O_2 uptake	Succinic dehydrogenase activity	Incorporation rate of P^{32} into phospholipid	Incorporation rate of P^{32} into residual fraction
Control		100 (%)	100 (%)	100 (%)	100 (%)
ATP	10^{-3} *M* (A)	111.0		25.6	22.1
Adenylic acid	10^{-3} *M* (A)	100.0		69.5	100.0
Phosphate	10^{-3} *M* (A)	98.0		13.1	0.5
Cytochrome c	5×10^{-4} *M* (A)			128.0	145.0
FAD	10^{-3} *M* (A)	103.0		100.5	98.1
Citrate	10^{-3} *M* (A)	101.0		110.0	128.0
Treated with CH_2ICOOH (10^{-2} *M*) for 4 hr. and washed +ATP	10^{-3} *M*	104.0		1.1	11.3
"+Adenylic acid	10^{-3} *M*	95.0		0.9	13.1
"+Phosphate	10^{-3} *M*	76.0		0.4	7.5
"+Cytochrome c	5×10^{-4} *M*	104.0		1.2	17.7
"+FAD	10^{-3} *M*	101.0		1.1	19.8
"+Citrate	10^{-3} *M*	100.0		1.1	15.2
CH_2ICOOH	10^{-2} *M* (B)	100.0	12.1	0.7	24.9
Treated with DNP (10^{-2} *M*) for 4 hr. and washed+ATP	10^{-3} *M*	87.0		70.0	69.7
DNP	10^{-2} *M* (B)	76.0		76.4	82.6
Treated with NaN_3 (10^{-2} *M*) for 4 hr. and washed+ATP	10^{-3} *M*	101.0		88.7	109.2
NaN_3	10^{-2} *M* (B)	79.0		71.5	89.3

A and B in the parenthes show the incubation methods.

Effects of Cofactors and Some Biochemical Agents—The effects of cofactors and some biochemical agents were tested and the results are shown in Table V. The results show that cytochrome c and citrate stimulated the incorporation of P^{32} into the phospholipid and the residual fractions. ATP and adenylic acid reduced the incorporation of P^{32} into the phospholipid fraction.

TABLE VI

Effects of Antituberculosis Agents and Antibiotics on the Incorporation of P^{32}

Antituberculosis agent and antibiotics, and incubation method		O_2 uptake	Succinic dehydrogenase activity	Incorporation rate of P^{32} into phospholipid		into residual fraction	
Control		100.0 (%)	100.0 (%)	100.0	(%)	100.0	(%)
Streptomycin	10^{-2} *M* (A)	106.0		75.9		888.8	
	(B)	100.0		90.7		99.8	
	(C)	98.0	15.7	97.0		15.1	
Chloramphenicol	10^{-2} *M* (A)	102.0		46.4		503.6	
	(B)	94.0		91.0		67.1	
	(C)	116.0	164.0	15.9		13.6	
Acriflavine	10^{-2} *M* (A)	59.0		39.7		28.9	
	(B)	17.0		101.4		39.0	
	(C)	43.3	38.5	14.7		5.8	
Acromycin	5 mg./ml. (A)	95.0		16.1		24.5	
	(B)	81.0		71.0		23.5	
	(C)	68.0	39.0	10.4		10.8	
INH	10^{-3} *M* (A)	93.0		106.5		90.0	
	(B)	140.0		205.0		70.1	
	(C)	85.5	65.0	165.0		82.5	
PAS	10^{-3} *M* (A)	103.0		117.2		83.8	
	(B)	116.0		455.0		280.0	
	(C)	87.5	70.0	164.0		255.0	
Terramycin	5 mg./ml. (A)	94.0		51.9		24.2	
	(B)	65.0		0.5		37.2	
	(C)	85.5	72.0	4.9		28.9	
Aureomycin	5×10^{-3} *M* (A)	98.0		11.9		27.4	
	(B)	32.0		33.4		9.3	
	(C)	24.7	40.5	0.9		0.6	
Promin	10^{-2} *M* (A)	122.0		136.8		91.5	
	(B)	98.0		57.7		40.2	
	(C)	119·5	159.0	3.6		0.7	
Thibion	17 μg./ml. (A)	86.0		35.6		61.9	
	(B)	99.0		204.0		92.4	
	(C)	140.0	147.0	73.5		101.0	
Tuberflavine	10^{-2} *M* (B)	88.0		176.3		50.3	
	(B)	105.0		120.3		102.8	

A, B and C in the parenthes show the incubation methods.

Inhibitory effect on the incorporation was observed by the addition of 10^{-3} *M* quinine (Table I), an inhibitor of certain flavin enzymes. The addition of flavin adenine dinucleotide (FAD) (10^{-3} *M*), however, had no effect on this process. This suggests that flavin enzymes containing FAD may not

participate in the P^{32}-incorporation, but other flavin enzymes, in which FAD is not concerned, or other enzyme systems which are inhibited by quinine may participate in the process.

The bacilli were treated with mono-iodoacetate for 4 hours at 37°, washed with the buffer, and incubated in the incubation medium containing citrate or cofactors such as cytochrome c, ATP, and adenylic acid, but the inhibitory effect of mono-iodoacetate was not reversed by the addition of citrate or cofactors. After treatment with 2,4-dinitrophenol or azide, and when ATP was added in the incubation media, the incorporation rate decreased.

Effects of Antituberculosis Agents and Antibiotics—The effects of various antituberculosis agents and antibiotics are shown in Table VI. Chloramphenicol reduced the incorporation of P^{32} into the phospholipid. It seems that this reduction was caused by the inhibitory effect of chloramphenicol on the energy-supplying system. Achromycin, terramycin and aureomycin also inhibited the incorporation of P^{32}. Isonicotinyl hydrazide (INH), p-aminosalicylic acid (PAS) stimulated the incorporation of P^{32} into the phospholipid. Some other agents caused a stimulation of the incorporation of P^{32}, but some others a reduction. Streptomycin slightly reduced the incorporation of P^{32} into the phospholipid, and markedly reduced the incorporation of P^{32} into the residual fraction in method C.

DISCUSSION

Dawson's investigation (*2*) showed that the incorporation of P^{32} into the phospholipid of brain dispersion required oxidizable substrate, oxygen, phosphate acceptor, magnesium ions and cytochrome c for optimum incorporation, and the presence of fluoride ions accelerated the incorporation of P^{32}. While the inhibitors of oxidative phosphorylation and of respiration inhibited it. Therefore, it was concluded that the initial step in the incorporation is an esterification of P^{32} into high energy phosphate compounds, which then phosphorylated phospholipid precursors. In the present investigation with the avian tubercle bacilli, the inhibitors of respiration and oxidative phosphorylation as well as SH-inhibitors reduced, while cytochrome c or citrate stimulated the incorporation of P^{32} into the phospholipid. The incorporation was reduced under the anaerobic condition. The effects of the inhibitors, inorganic phosphate, ATP, cytochrome c and anaerobic condition were similar to Dawson's results (*2*). These results indicate that the process of P^{32}-incorporation into the phospholipid is associated with oxidative phosphorylation and TCA cycle, SH-groups and cytochrome c taking part in this process. The addition of cysteine reduced the rate of P^{32}-incorporation in the system treated with PCMB or monoiodoacetate. It has been known that cysteine is oxidized by the avian tubercle bacilli*; the evolution of H_2S could be detected by the smell during the incubation. H_2S is known to be an inhibitor of the incorporation of P^{32}

* Hoshino, M. Catabolism of lecithin and lysolecithin by the avian tubercle bacilli. Personal communication.

into the phospholipid of liver and kidney (*22*). From these facts it is presumed that cysteine added was attacked by the bacilli, and H_2S thus evolved inhibited the incorporation of P^{32}.

Sugibayashi *et al.* (*1*) reported that streptomycine had no effect on the incorporation of P^{32} into the lipid of the avian tubercle bacilli but inhibited the incorporation of P^{32} into the ribonucleic acid fraction. In our experiment with method C, streptomycine apparently reduced the incorporation of P^{32} into the residual fraction. This result suggests that streptomycine inhibits the incorporation of P^{32} into the nucleic acid fraction such as ribonucleic acid.

In the present investigation the behaviors of the intact tuberble bacilli upon the synthesis of phospholipid were observed. However, it has been known that the phospholipid is synthesized in the mitochondria from rat liver (*8–12*), and enzymatic synthesis of lecithin and phosphatidyl ethanolamine are mediated by cytidine coenzymes (*12*). And Kornberg (*13*, *14*) demonstrated that the enzyme preparation isolated from liver combined long chain fatty acid and α-glycerophosphate. Therefore, the observation of the enzymatic reaction with the mitochondria from the tubercle bacilli will be required for the detailed investigation of the mechanism of the phospholipid synthesis.

SUMMARY

The incorporation of P^{32} into the phospholipid and the residual fractions of avian tuberble bacilli was studied under various conditions. By the addition of inorganic phosphate and under anaerobic condition, the incorporation rate of P^{32} was reduced. The incorporation rate increased after treating the bacilli in veronal buffer for 4 hours at 37°.

The effect of various inhibitors, cofactors, antibiotics metal ions upon P^{32}-incorporation was also investigated and the mechanisms of the process were discussed.

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TRYPSINOGEN-KINASE IN ASPERGILLUS ORYZAE

VI. ON ITS SUBSTRATE SPECIFICITY AS PROTEASE

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The author (*1*) previously reported the followings: (a) In the case of trypsinogen-activation by trypsinogen-kinase, Val-$(Asp)_4$-Lys was liberated and trypsin containing isoleucine as a N-terminal was produced, similar to the case of activation by trypsin (*2*) or by enterokinase (*3*); (b) The liberated Val-$(Asp)_4$-Lys was partially hydrolysed to give Val, $(Asp)_4$ and Lys, but $(Asp)_4$ was not further hydrolysed. The substrate specificity of trypsin has been investigated by Bergman and his associates (*4*) and summarized by Neurath (*5*). It is well known that the amide bonds of benzoyl-Arg-NH_2 and benzoyl-Lys-NH_2 are hydrolysed but that of benzoyl-ε-Cbz-Arg-NH_2 is not. The basic group of Arg and Lys then appears to be necessary for the hydrolysis. It is not possible to explain in full the trypsinogen-activation if based only upon the substrate specificity of trypsin, but if the linkage between Lys and iso-Leu in trypsinogen is presumed to be particularly susceptible to hydrolysis, *e.g.* if it is placed near the end of the trypsinogen molecule or on its surface, the hydrolysis of this linkage by trypsin would be comprehensible, to some extent, by its substrate specificity. Kawaharada (*6*) found that trypsinogen was activated to trypsin by an enzyme from animal tissues which seemed to be cathepsin. Fruton (*7*) observed that cathepsin B also hydrolysed bezoyl-Arg-NH_2. The same explanation could be given.

What kind of substrate specificity is responsible to the activation by trypsinogen-kinase? In other words, how can the mechanism of activation be explained by the properties of this protease? In the previous report (*1*), the some proteolytic properties of this enzyme were studied, but any specific properties, which provide some suggestion on this activation mechanism, could not be disclosed. The present report is described on this problem.

EXPERIMENTALS

Experiment I. Hydrolysis of Benzoyl-Arg-NH_2 and Activation of Trypsinogen

Experiments were carried out to observe whether trypsinogen-kinase has the same substrate specificity as trypsin, *i.e.* it can hydrolyse benzoyl-Arg-NH_2. At the same time, experiments with proteases supplied by various investigators were made. The used proteases were as follows:

Protease from Streptomyces griseus (*8*): Paper-electrophoretically homogeneous powder—this enzyme is able to hydrolyse the peptide bonds of casein to the extent of 80–90 per cent.

Alkaline-protease from Aspergillus oryzae (*9*, *10*): Paper-electrophoretically homogeneous powder—this enzyme and trypsin exhibit similar actions in the hydrolysis of casein but the substrate specificity of the former is unknown.

Neutral-protease from Aspergillus oryzae (*10*): Paper-electrophoretically homogeneous powder—metallic ions are necessary for its enzymatic activity but the substrate specificity is unknown.

Acid-protease from Aspergillus oryzae (*1*): Paper-electrophoretically homogeneous powder—this enzyme is the trypsinogen-kinase.

Protease from Bacillus subtilis (*11*): Crystals (Nagase Preparation)—the denatured proteins are very rapidly and completely hydrolysed to TCA soluble components by this enzyme but the substrate specificity is unknown.

Acid-protease from Aspergillus saitoi (*12*): Crystals—this enzyme resembles the acid-protease from *Asp. oryzae* in its ability of casein-hydrolysis. It readily hydrolyses Cbz-L-Glut-L-Tyr, the specific substrate of pepsin, and benzoyl-Arg-NH_2 is hydrolysed in the presence of an enormous amount of this enzyme.

Trypsin: Crystals (G.B.I.)

Using the above 7 proteases at their respective optimum pH's, degree of hydrolysis of benzoyl-Arg-NH_2 and trypsinogen-kinase-like activity were determined. The results are given in Table I. Only trypsin hydrolysed benzoyl-Arg-NH_2, and the other proteases exhibited no hydrolytic action, even in the use of the same amounts of the enzymes. However, both the acid-protease from *Asp. oryzae*, *i.e.* the author's trypsinogen-kinase, and the acid-protease from *Asp. saitoi*, strongly activated trypsinogen. These results show, at least, that no direct proportional relationship exists between the activities of hydrolysing benzoyl-Arg-NH_2 and of activating trypsinogen.

Experiment II. Activation of Trypsinogen by Neutral-Protease

It is interesting that the neutral-protease from *Asp. oryzae* activates trypsinogen to the same extent as trypsin, although it does not hydrolyse benzoyl-Arg-NH_2. However, the probability of contamination of the acid-protease into the preparation of the neutral-protease should be taken into consideration. It is known that ethylenediamine-tetraacetic acid (EDTA) inhibits activity of the neutral-protease but not that of the acid-protease when casein and other proteins are used as the substrate. Inhibitory action of EDTA at pH 5.5 was investigated. The acid-protease can still, although weakly, activate trypsinogen at pH 5.5. As shown in Table II, the activation of trypsinogen by the neutral-protease is also completely inhibited by EDTA while the activation by the acid-protease is not inhibited at all. Therefore, it was found that the neutral-protease also, although weakly, activates trypsinogen.

Experiment III. Hydrolysis of Synthetic Peptides, Especially Those Containing Arginine

Although this enzyme does not hydrolyse benzoyl-Arg-NH_2 as shown in

Table I

Hydrolysis of Benzoyl-Arg-NH_2 and Activation of Trypsinogen by Various Proteases

Proteases	pH	Hydrolysis of Bz-Arg-NH_2	Activity of the kinase		
			Concentration of Enzyme 0.1 μg./ml.	4 μg./ml.	20 μg./ml.
Trypsin	8.0	84 (%)	—	0.005	0.058
St. griseus	8.0	0	—	—	0.011
B. subtilis	8.0	0	—	—	—
Asp. oryzae (alkaline)	8.0	0	—	—	—
Asp. oryzae (neutral)	5.5	0	—	0.179	+
Asp. oryzae (acid)	3.0	0	0.180	+	+
Asp. saitoi (acid)	3.0	0	0.260	+	+

Hydrolysis of Bz-Arg-NH_2:

Bz-Arg-NH_2	3×10^{-2} *M*	1 ml.	After 24 hrs. at 30°, NH_3 was measured by Conway's microdiffusion method. (*13*)
Buffer solution		1 ml.	
Protease 200 μg./ml.		1 ml.	

Activity of the kinase:

Trypsinogen	1 mg./ml.	0.5 ml.	Activation for 1 hr. at 35°	The determination of trypsin-activity was done as shown in previous paper. (*1*)
Protease in buffer solution		0.5 ml.		
NaOH for neutralization or H_2O		1 ml.	The mixture 2 ml.	
2 per cent casein in *M*/10 Na_2HPO_4		1 ml.		

Table II

Comparison of Acid-Protease and Neutral-Protease in Trypsinogen Activation

	Acid-protease		Neutral-protease	
Concentration of protease	8 μg./ml. pH 5.5 buffer solution		8 μg./ml. pH 5.5 buffer solution	
Protease solution	1 ml.	1 ml.	1 ml.	1 ml.
EDTA (2×10^{-2} *M*) (+) or water (−)	(−) 1 ml.	(+) 1 ml.	(−) 1 ml.	(+) 1 ml.
After the mixtures were left standing for 1 hr. at room temperature, the activity of the kinase was determined by the method as shown in Table I.				
The activity of the kinase	0.150	0.165	0.110	0.001

Experiment I, the Lys-iso-Leu bond and the Asp-Lys bond are hydrolysed in the process of trypsinogen-activation by this enzyme. Therefore, it seems to have some affinity towards basic amino acids. On the other hand, it appears that the protected N-terminal and the free C-terminal are necessary for the peptides to be hydrolysed, because, as reported previously (*1*), only Cl-acetyl-L-Leu and Cbz-DL-Ala-Gly-L-Leu in various peptides, are hydrolysed while free peptides are not attacked. Hydrolysis by the acid protease of the 11 peptides listed in Table III were performed under the same conditions as shown in the preceding paper (*1*). The six peptides containing arginine are synthetized at Ando Laboratory of Tokyo University and the others are the preparation of G.B.I. As shown in Table III, DL-Ala-Gly-Gly, L-Leu-Gly-Gly, L-Arg-L-Leu, L-Arg-L-Phe and benzoyl-Arg-NH_2 were not affected at all.

TABLE III

Hydrolysis of Synthetic Substrates by Acid Protease

Substrates	Final concentration (10^{-3} *M*)	Hydrolysis (%)
DL-Ala-Gly-Gly	2	3
L-Leu-Gly-Gly	2	0
Cbz-L-Glu-L-Tyr	0.6	90
L-γ-Glu-L-Cys-Gly	2	36
Cbs-L-Leu-L-Arg	1	99
L-Leu-L-Arg	1	72
Bz-L-Arg-L-Leu	1	56
L-Arg-L-Leu	1	5
L-Phe-L-Arg	1	45
L-Arg-L-Phe	1	0
Bz-L-Arg-NH_2	2	0
Bz-L-Arg	2	0

Hydrolysis was performed under the same conditions as shown in the preceding paper (*1*).

Cbz-L-Glut-L-Tyr, Glutathione, Cbz-L-Leu-L-Arg, benzoyl-L-Arg-L-Leu, L-Leu-L-Arg and L-Phe-L-Arg were hydrolysed. Assuming that, glutathione, having γ-glutamyl, is a peptide containing a protected N-terminal, glutathione, Cbz-L-Glut-L-Tyr, Cbz-L-Leu-L-Arg and benzoyl-L-Arg-L-Leu may be classified as the peptides containing a protected N-terminal and free C-terminal. These facts well support the hypothesis stated above.

Experiment IV. The C-terminal Amino-acid of Trypsinogen

The hydrolysis of Cbz-L-Leu-L-Arg, as shown in Experiment III, is a very characteristic property of this enzyme. The C-terminal amino-acids of trypsinogen and trypsin are not hydrolysed by carboxypeptidase (*14*) and they are considered to be either acidic or basic amino-acids. Recently, Gladner (*15*) reported that the C-terminal was not detected by using carboxypeptidase B. The author, however, presumed possibility of liberation of a basic amino-acid from the C-terminal of trypsinogen in the process of trypsinogen-activation by this enzyme, and the following experiment was performed. One hundred mg. of trypsinogen was dissolved in 10 ml. of diluted Walpole buffer solution (1 : 50) at pH 3.5. The solution was treated with *ca.* 300 μg. of trypsinogen-kinase for 4 hours at 0° to complete activation. Ten ml. of 10 per cent TCA was added to the activated solution, and the mixture was left standing for 1 hour at 35°, followed by centrifugal separation. The precipitate obtained was washed twice with 10 ml. portions of 5 per cent TCA, and the washings were mixed with the previously separated supernatant and then filtered through a stiff filter paper. About 30 ml. of the filtrate was shaken with an equal volume of ether to remove the TCA. Dowex-1 × 4 (HCO_3-type) was added to make the solution slightly alkaline. The resin was filtered, washed with water, and the filtrate was evaporated to dryness under reduced pressure. The obtained sample was dissolved in a small amount of water and the solution was subjected to paper chromatography with the following 5 solvents: (a) butanol : acetic acid : water = 4 : 1 : 5, (b) butanol : acetic acid : water = 4 : 2 : 1, (c) phenol : water = 8 : 2, (d) phenol : water : 80 per cent formic acid = 30 : 10 : 0.4, (e) phenol : 1 per cent NH_3aq. = 4 : 1. There appeared only one spot corresponding to arginine in every case, with which Sakaguchi's test was also positive. Microbiological assays with *Lactobacillus casei*, *Lactobacillus fermenti*, *Leuconostoc mesenteroides* and *Streptococcus faecalis* indicated the presence of arginine.

DISCUSSION

It is well known that trypsin has a particular affinity to arginine and lysine. The author's enzyme preparation hydrolyses Cbz-L-Glut-L-Tyr, glutathione, Cbz-L-Leu-L-Arg, benzoyl-L-Arg-L-Leu, Cbz-DL-Ala-Gly-L-Leu, L-Leu-L-Arg and L-Phe-L-Arg, while it does hydrolyse neither free peptides nor benzoyl-Arg-NH_2. The most remarkable properties of this enzyme preparation are as follows:

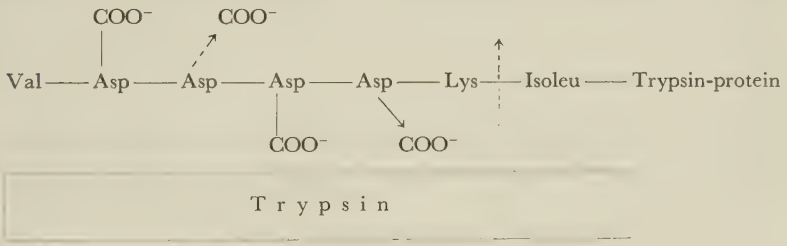
The first is the hydrolysis of Cbz-L-Leu-L-Arg. Enzyme which hydrolyses the peptides containing protected N-terminal and free C-terminal, such as benzoyl-L-Arg-L-Leu, is generally named carboxypeptidase-A. However, carboxypeptidase-A shows its activity only when the C-terminal is a neutral amino-acid but not when it is an acidic or basic amino-acid. Folk (*16*) has recently discovered an enzyme which hydrolyses peptides only when the C-terminal is a basic amino-acid and named it basic carboxypeptidase or

carboxypeptidase-B. This enzyme readily hydrolyses benzoyl-Gly-L-Arg and benzoyl-Gly-L-Lys. However, when the C-terminal is a neutral amino-acid, such as Cbz-L-Arg-Gly-L-Leu, hydrolysis does not take place. The fact that the author's enzyme hydrolyses both Cbz-L-Leu-L-Arg and benzoyl-L-Arg-L-Leu suggests that it has both properties of carboxypeptidases-A and -B. The second property is that L-Leu-L-Arg and L-Phe-L-Arg are both hydrolysed although no other free peptides in general are affected. In other words, free peptides are also hydrolysed only when they have the C-terminal of basic amino-acids.

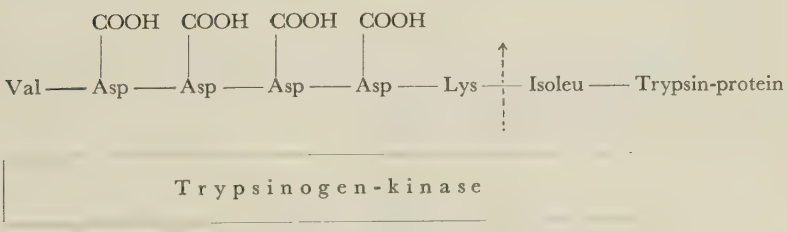
From the above observations, it is conceivable that this enzyme exhibits a specific affinity towards arginine and basic amino acids. The Lys-iso-Leu bond of trypsinogen is hydrolysed by this enzyme owing to this affinity.

The separation of lysine from the liberated Val-$(Asp)_4$-Lys can be naturally inferred from the fact that L-Leu-L-Arg, L-Phe-L-Arg and Cbz-L-Leu-L-Arg are hydrolysed by this enzyme. On the other hand, hydrolysis of Val-Asp bond is not explainable on the basis of substrate specificity. That this enzyme cannot hydrolyse $(Asp)_4$, which has an opposite property to that of basic amino-acids, can be explained to some extent by the above consideration.

Rate of trypsinogen activation by trypsinogen-kinase is much greater than that by trypsin. Difference in enzymatic properties of trypsin and



The activation by trypsin at pH 8.0



The activation by trypsinogen-kinase at pH 3.5

FIG. 1. Schemata in the activation of trypsinogen by trypsin and trypsinogen-kinase.

trypsinogen-kinase themselves would be mainly responsible for this phenomenon, but the following factor can also be conceivable on the side of trypsinogen. According to Neurath (2), the isoelectric point of Val-$(Asp)_4$-Lys is pH

3.5. Therefore, in the case of activation by trypsin at pH 7–8, $(Asp)_4$ is completely dissociated and the four COO^- groups in $(Asp)_4$ are electrostatically repulsed by one another and extend out in a brushlike form as shown in Fig. 1. On the contrary, this dissociation is suppressed in the case of activation by trypsinogen-kinase at pH 3-4, and the configuration of this group is flat or contractive. Therefore, in the author's opinion, this projection of COO^- would inhibit approach of trypsin and trypsinogen, whereas this inhibition does not occur in the case of trypsinogen-kinase and approach of trypsinogen and trypsinogen-kinase takes place freely, resulting in very rapid activation.

The enzyme preparation used in this study had the properties of carboxypeptidase A, B and proteinase. Whether these enzymatic activities were due to mixing of enzymes or to one has not been verified, but the greater part of the observed actions, apparently, seemed to be brought about by one enzyme.

SUMMARY

1. Hydrolysis of benzoyl-Arg-NH_2 and the activity of trypsinogen-activation by the trypsinogen-kinase and other various proteases were examined. Both the trypsinogen-kinase (acid-protease from *Asp. oryzae*) and the acid-protease from *Asp. saitoi* could not hydrolyse benzoyl-Arg-NH_2 but activated trypsinogen strongly.

2. The neutral-protease from *Asp. oryzae* has also a weak activity in trypsinogen-activation.

3. The preparation of the author's acid-protease has the activities of carboxypeptidase A, B, proteinase, and of hydrolysing L-Leu-L-Arg and L-Phe-L-Arg.

4. The relation between the substrate specificity of the acid-protease from *Asp. oryzae* and the mechanism of trypsinogen-activation by this enzyme are discussed.

The author wishes to express his thanks to Prof. Akabori of Osaka University for his kind guidance, and to Mr. Nomoto, Mr. Miura and Dr. Yoshida for supplying the used proteases, and to the staff of the Ando Laboratory of Tokyo University for giving the arginine containing peptides.

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STUDIES ON ENZYMATIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUES*, **

I. FUNDAMENTAL PROPERTIES OF THE REACTION

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Mechanism of the biosynthesis of cocarboxylase has been the subject of much conjecture and various hypotheses to account for it have so far been presented as follows: i) a "one-step" transpyrophosphorylation, T+ATP → TDP+A5P, proposed by Weil-Malherbe (*1*) and Leuthardt and Nielsen (*2*): ii) a "two-step" transorthophosphorylation, T+ATP → TMP+ADP, TMP+ADP → TDP+ A5P (and also T+ADP → TMP+A5P, TMP+ATP → TDP+ADP), as has been reported by Thoai *et al.* (3); and other special cases: iii) T+2ATP → TDP+2ADP (*4*), and iv) T+2P → TDP+$2H_2O$ (*5*) : v) TMP+PP → TDP+P (*6*). The latter two reactions could occur only under non-physiological conditions and the synthetic mechanisms may be based on the reversal reaction of phosphatase. However, as none of these theories is based on the experimental evidence enough to draw any conclusion, an extensive reexamination is necessary to decide which is the real process. The solution of the problem implies also the clarification of a possibility of biological transpyrophosphorylation as has recently been suggested by Kornberg and his collaborators (*7*, *8*) in phosphoribosyl pyrophosphate formation.

The present paper shows the character of enzymatic constitution of the synthetic system, fundamental properties of the reaction, and evidences upon which is based the assertion of the mechanism of the above theory (i).

A direct proof and more detailed study of the mechanism and other relevant studies will be reported in later papers.

METHODS AND MATERIALS

Enzyme—The enzyme which was designated as thiaminokinase (*9*) was prepared from rat liver for this purpose. The enzymes used throughout this experiment were prepared

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** The following abbreviations are used throughout this paper: ATP, ADP, and A5P, adenosine tri-, di-, and monophosphate-5′; G5P, I5P, and R5P, guanosine-, inosine-, and ribose -phosphate-5′; A3P, G3P, U3P, and C3P, adenosine-, guanosine-, uridine-, and cytosine -phosphate-3′; PEP, phospho (enol) pyruvate; TDP and TMP, thiamine pyro (di)- and monophosphate; T, thiamine; P and PP, inorganic ortho- and pyrophosphate; Tris, tris (hydroxymethyl)-aminomethane.

according to the method of Leuthardt and Nielsen (*2*) up to the step of "precipité HCl" (Prep. A) unless otherwise stated. In some cases, purification was made along their method to the ammonium sulfate fractionation (Prep. B) and further acetone fractionation (fraction between 37 and 48 per cent, Prep. C) was added as the third step. The enzyme used in the phosphate-free system was prepared by a similar way, but Tris buffer (0.05 *M*, pH 7.4) was used instead of phosphate buffer.

Standard Condition for Enzyme Assay—The following standard condition was adopted unless otherwise specified. The reaction mixture contained: 10^{-4} *M* thiamine, 3.3×10^{-3} *M* $MgSO_4$, 10^{-3} *M* of phosphate-donor compounds, 2.3 or 2.8×10^{-2} *M* phosphate or Tris buffer of pH 7.4*, and the enzyme solution which contained 4-6mg. of protein. Total volume was made to 3.0 ml. The above reaction mixture was incubated at 37° for 60 minutes in an air phase. Then the reaction was stopped by heating at 100° for one minute in a boiling water bath. It was ascertained that the amount of TDP was preserved quantitatively during this treatment. Immediately after deproteinization the reaction mixture was diluted with water, the pH was adjusted to 6.0 with HCl, and the resulted solution was submitted to the determination of TDP synthesized.

For this purpose TDP to be tested was recombined with apocarboxylase and Mn^{++}, and the carbon dioxide evolved from pyruvate was measured manometrically. Apocarboxylase used in this experiment was obtained by Kaziro's method (*10*). The purified apocarboxylase retained ordinarily the activity 1,000 to 2,000 in Q_{CO_2} and showed no effect on the addition of thiamine (*11*). The vessels for the determination contained: 6.7×10^{-2} *M* sodium pyruvate; 10^{-2} *M* $MnCl_2$; 1.0 ml. of TDP to be estimated; 8.3×10^{-2} *M* citrate buffer (pH 6.0), and 0.2 ml. of purified apocarboxylase. Total volume was made to 3.0 ml. This mixture was incubated at 30° for 30 minutes. The amount of TDP was usually indicated in μl. of carbon dioxide evolved in 30 minutes for 1/5 to 1/20 aliquot of the mixture, and the values were corrected in a similar way to Singer and Pensky (*12*). Consequently, under an appropriate condition these values indicate a measure of relative amount of TDP in one experimental series. If needed, however, calibration curve was made by a standard TDP and the absolute amount of TDP was evaluated. Under the condition studied, the carboxylase activity was neither inhibited by the presence of A5P or ADP nor accelerated by the presence of ATP or PEP. The effects of added substances on the carboxylase were subtracted in all the results shown in the text.

Determination of ATP—ATP was assayed by use of hexokinase (*13*).

Separation of the Reaction Products—(*i*) *Paper Chromatography*: The reaction mixture was cooled in ice bath and deproteinized by addition of an equal volume of ice-cold 15 per cent perchloric acid. Then it was neutralized to pH 7.0 with dilute KOH and, after separation of precipitated potassium perchlorate by centrifugation, 1/10 volume of 20 per cent mercury acetate (dibasic) was added to the supernatant at pH 3.5. The precipitate formed on standing was spun down, suspended in a small amount of water, and subjected to H_2S degradation. After centrifugation and aeration the clear supernatant was dried from frozen state. This was dissolved in a minute amount of water and after treatment cation exchange resin, Amberlite IR-120 (H^+), the resultant matter was used as the chromatographic sample. Paper chromatography was carried out by ascending method using Toyo filter paper No. 53 at 2-4°. The developing solvent was composed of *n*-butanol, isoamyl alcohol, water, isobutyric acid, and ammonia water (28 per cent) in a

* Although the optimum pH of the enzyme was revealed to be 8.4 as will be described in a later paper, the reaction condition was selected usually at pH 7.4 in view of the physiological condition.

ratio of 7.2 : 2.5 : 7.5 : 12.0 : 0.2 parts by volume. (*ii*) *Column chromatography*: The reaction mixture was treated in a similar way as described above up to the step of deproteinization. The filtrate after separation of potassium perchlorate was extracted three times with equal volume of the mixture of water-saturated phenol and chloroform in a ratio of 9 : 1 by volume, which was warmed to 35° in advance. Then the combined extract was again shaken with equal volume of ether. In the resulting aqueous layer thiamine derivatives and nucleotides in the original extract were concentrated in a small volume and the solution was almost free from inorganic salts which may interfere the charge of weak electrolytes such as TMP or TDP against the column. All the activity of TDP was found in this fraction. This aqueous layer was further extracted twice with an equal volume of ether in order to remove phenol completely. Then ether was evaporated from this layer by a sufficient aeration and the aqueous solution was passed through the column of Dowex-1 (Cl^-) at pH 8.0. For the elution HCl-NaCl system was applied in a similar way to that of Cohn and Carter (*14*).

Materials—ATP (crystalline), ADP, and A5P were obtained from the Sigma Chemical Company and the Pabst Laboratories. ATP and ADP were used as sodium salt, and each of them was ascertained to be chromatographically pure. No contamination of ATP in the preparation of ADP was confirmed by glycerinized muscle contractability, and absence of A5P in the same preparation was made sure by adenylic deaminase system, and no detectable impurities, such as ADP and ATP, in the preparation of A5P, was checked by myokinase-adenosinetriphosphatase system. PEP was synthesized by the method of Baer (*15*), TMP was prepared according to the method of Karrer and Viscontini (*16*), and TDP was donated by the Takeda Pharmaceutical Industries, and was 92 per cent by manometry. The purity of TMP is described in the section on experimental results. Other phosphate compounds were purchased from the Nutritional Biochemicals Corporation and the Schwartz Laboratories. Radioactive pyrophosphate was prepared by the method of Kornberg and Pricer (*17*).

RESULTS

Essential Factors for TDP Formation—The essential factors for *in vitro* synthesis of TDP were proved to be thiamine, phosphate donor such as ATP, and divalent cations such as Mg^{++} or Mn^{++}. In the absence of any of them, no synthesis of TDP was observed (Table I).

TABLE I

Essential Factors for the Reaction

Factors added, in final concentration				
Thiamine (10^{-4} *M*)	ATP (10^{-3} *M*)	MgCl (3.3×10^{-3} *M*)	Enzyme	Activity (μl. of CO_2)
+	+	+	+	206
−	+	+	+	4
+	−	+	+	0
+	+	−	+	7
+	+	+	−	0

The reaction condition and the method for assay followed the description under the Method except omissions in specified cases.

Inorganic phosphates, such as orthophosphate or pyrophosphate reported by Steyn-Parvé (*9*) and Thai *et al.* (*5*, *6*), had no direct connection with this reaction except metaphosphate, which is a strong inhibitor as a protein coagulant. As the enzyme preparation used in this work was almost free from inorganic phosphate, this fact suggests that under physiological condition there is no possibility of direct phosphorylation by inorganic phosphates. It was also confirmed from the experiment using radioactive orthophosphate and pyrophosphate observing no incorporation into the TDP formed under the condition studied.

There is one fact which must be noted here: On dialyzing the enzyme preparation against water, a marked decrease of the activity was observed. The concentrated dialysate was substituted almost completely with ammonium sulfate, sodium chloride, or other neutral salts for recovery of the activity. Among such complementary factors, so far as were tested, ammonium sulfate was the most effective.

Time Course of the Reactions—Experiments were carried out with several phosphate compounds to test their activities as a phosphate donor. ATP, ADP, and the pair of PEP and A5P, which may show representative different types in the mechanism, were chosen for the phosphate donor as model reactants. The time course of TDP formation in the above-mentioned reactions was observed as shown in Fig. 1. Each of the reactions with ATP, ADP, and the pair of PEP and A5P were characterized by their time courses. For

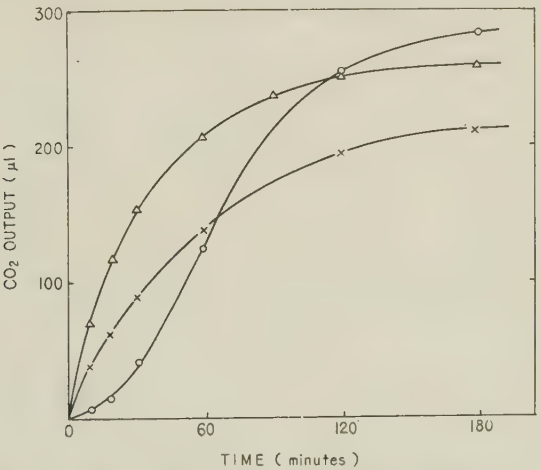

FIG. 1. Time courses of the three typical reactions. —△— ATP, 10^{-3} *M*; —×— ADP, 10^{-3} *M*; —○— PEP (2×10^{-3} *M*) coupling with A5P (10^{-3} *M*).

ATP-reaction, the formation proceeded linearly in the initial 30 minutes, then decreased gradually, and after 150 minutes the formation of TDP was hardly observed. The latter two reactions, some composite enzyme reactions as will be stated later, proceeded in different ways. There was no lag phase in the

process of ADP, in contrast to the pair of PEP and A5P with a marked lag phase. As can be seen in the above results, it is noted that the reaction is of sluggish nature and may be influenced by a number of rapid reactions, and the amount of TDP synthesized does not go beyond a certain limit.

Relationship between ATP and ADP for the Reaction—To elucidate the mechanism of transphosphorylation, the activities of phosphorylation of ATP and

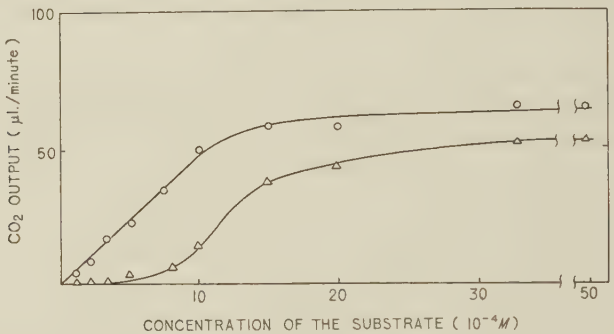

FIG. 2. Concentration dependence in initial velocities with ATP and ADP.

Incubation period was designed for 15 minutes. —○— ATP, —△— ADP.

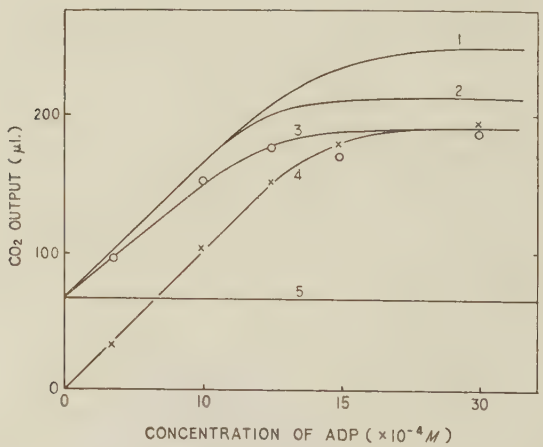

FIG. 3. Effect of the addition of ADP to a constant concentration of ATP. Curves 1 and 2, theoretical curves based on alternative pathways postulated as shown in the text; Curve 3, experimental data for the addition of ATP-system; Curve 4, data for the ATP-system; Curve 5, base line of the system of ATP at $4.5 \times 10^{-4} M$.

ADP were compared by the initial velocity. As shown in Fig. 2, TDP formed is proportional to the concentration of ATP when the latter is lower than

the concentration needed for the maximal velocity, while in the case of ADP, the curve follows practically a second order curve in the concentration below that for the point of inflexion. These findings show that ATP acts as a first-order reaction regarding itself but ADP behaves as a second-order one concerning itself. A part of this relationship would be supported by the fact that the critical concentration, minimum concentration to reach the maximal velocity, of ATP is approximately one-half of that of ADP. Although the order of reaction does not always correspond to the number of molecular reaction, these two agree with each other in almost all the reactions without complicated mechanism. Thus, ATP and ADP may take part in their reactions as one and two molecular units, respectively.

Another experiment was performed on the effect of ADP to a low constant concentration of ATP, 4.5 x 10^{-4} *M*, and the curve for ATP+ADP-reaction was obtained (Fig. 3). If the both of the reactions with ATP and ADP take the same final transphosphorylation pathway, curve 1 should be obtained theoretically, while if they take different routes, curve 2 should be obtained, so long as no interference exists between ATP and ADP. The result obtained, however, did not fit into any of the theoretical values and showed a somewhat depressed activity compared to the theoretical values. These facts suggest that ATP- and ADP-reaction would proceed through the same phosphorylation pathway, but ADP itself may show an inhibition to the phosphorylation with ATP in nature.

Effect of Adenylic Acid and Their Related Compounds—The inhibitory effect of A5P against the reaction with ATP and its ineffectiveness as a substrate was ascertained, but, as shown in Table II, it was found that this phenomenon occurred only with 5′-nucleotides, and other various 3′-nucleotides acted neither as an inhibitor nor phosphate donor. When Lineweaver-Burk's curve (*18*) was plotted, the inhibition of A5P was revealed to be a competitive one with $K_i = 3.6 \times 10^{-4}$ *M* as shown in Fig. 4. There is a possibility that the A5P inhibition is a drawback of ATP to ADP due to adenylate kinase reaction, A5P+ATP → 2ADP, as the product ADP is not a direct phosphate donor but is an inhibitor as will be indicated below. Although this possibility may not be excluded, the above experimental data showed that the inhibition, at least in the initial stage, is of a typical competitive nature. However, at a later stage of the reaction the effect of ADP formed may aggravate the inherent inhibition of A5P.

Thiamine and Thiaminemonophosphate as a Substrate—As shown in Fig. 5, one may point out that the critical concentration of thiamine holds surprisingly low and the Michaelis constant is 4.5×10^{-7} *M* in contrast to the order of 8.3×10^{-4} *M* for ATP. The possibility of TMP being an intermediate in this reaction would be excluded by the fact that it scarcely has an activity with ATP or ADP. TMP should be reasonably more effective than thiamine on coupling with ATP or ADP if it were the intermediate. Nevertheless, these data do not serve as a conclusive evidence for the denial of TMP acting as a substrate in this reaction. In coexistence of TMP and thiamine in

TABLE II

Effect of Adenine Nucleotides and Relevant Compounds on the Activity

Additions as nucleotide (10^{-3} *M*)	Activity (μl. CO_2)	Inhibition (per cent)
Exp. 1		
ATP	288	—
ATP+A5P	136	53
ATP+I5P	121	58
ATP+G5P	140	52
ATP+A3P	293	0
ATP+G3P	272	5
ATP+U3P	268	7
ATP+C3P	288	0
ATP+Adenosine	292	0
ATP+Adenine	298	0
ATP+R5P	292	0
Exp. 2		
ATD	214	—
ADP	7	—
ATP+ADP	153	28

Reaction condition followed the description of the standard condition except the addition of nucleotides as specified. The enzyme used for the experiments 1 and 2 were preparations A and C, respectively, illustrated in Fig. 9.

the same molar concentration near the critical concentration of thiamine, on the other hand, TMP showed some inhibitory effect as indicated in Fig. 5. The inhibitory action of TMP on the thiamine-reaction is a separate problem from its qualification as a substrate, but it may give a useful reference on the subject. If TMP does not act as a substrate, the question arises where the cause of the hitherto reported activity of TMP (*3*, *9*) comes from. The following experiments were carried out additionally: TMP had no TDP-like activity on coupling with apocarboxylase and the enzyme preparation had no activity which decomposes TMP to thiamine, such as the activity of thiamin pyrophosphatase (*19*, *20*). Hence it is reasonable to assume that the hitherto observed activity of TMP was caused from a minute contamination of thiamine that could not be detected by the ordinary analytical method, and this interpretation is strongly supported by the fact that Michaelis constant of thiamine in the reaction is very low.

Material Basis of the Representative Reactions—The above-mentioned experiments lead to the conclusion that the ATP- and ADP-reaction might have a common final step, and this deduction was also supported by the fact that the reaction could proceed with PEP in combination with A5P only, but not

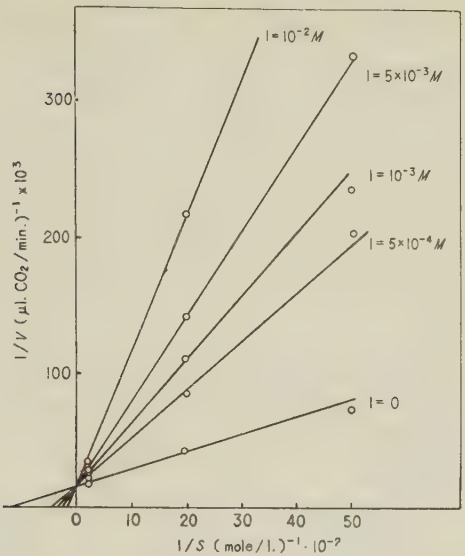

FIG. 4. Lineweaver-Burk's plot of A5P inhibition. Reaction period, 15 minutes. Preparation B was used as the enzyme.

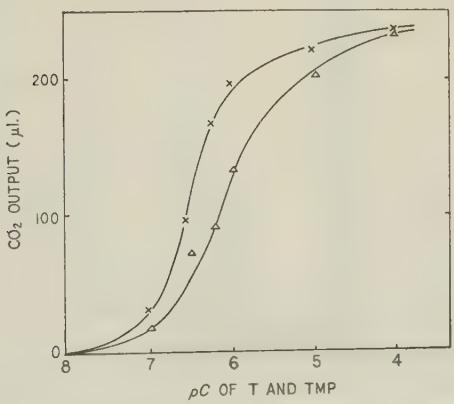

FIG. 5. Effect of TMP on T-system. Incubation period, 15 minutes.

with other nucleotides such as A3P or I5P. The chromatographic analyses indicated in Fig. 6 showed that no contradiction to the conclusion could be found. The expectation that the ADP- and PEP+A5P-reactions might be based on the same mechanism as ATP in their final steps was supported by the result shown in Fig 6. This figure shows the paper chromatographic proof of the formation of ATP in the case of ADP- and PEP+A5P-reactions regardless of the presence of thiamine in the reaction mixture. On the other hand, no synthesis of TDP was observed unless the formation of ATP was

provided. This fact shows that in this enzyme preparation an enzym is

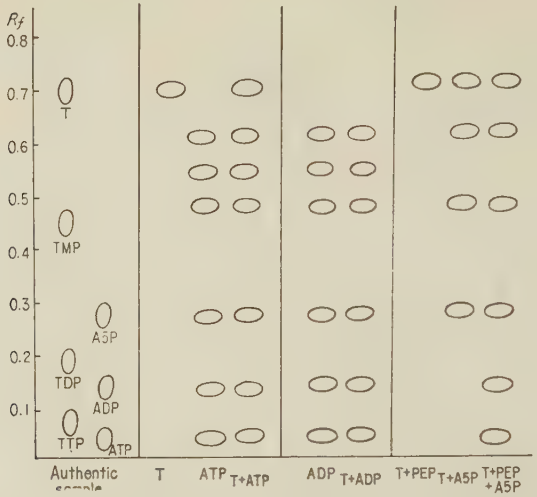

FIG. 6. Formation of ATP in ADP- and PEP+A5P-system. Procedure of the chromatography is described in the Method.

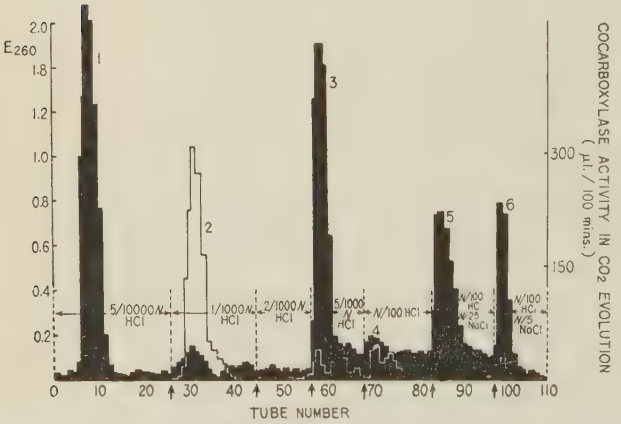

FIG. 7. Fractionation of the reaction products. The reaction was fortified by the addition of PEP ($2\times10^{-3}\,M$) as an ATP-regenerator. Column size, 1.0×7.0 cm; elution velocity, 0.8 ml. per minute. Other conditions were the same as described under the Method.

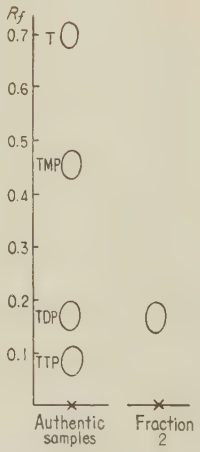

FIG. 8 Detection of TDP from the fraction 2 separated by Dowex-1 column.

Spot tested was positive to cocarboxylase activity and thiochrome test after hydrolysis and in agreement with authentic sample in ultraviolet absorption spectrum.

present which produces ATP besides the enzyme to phosphorylate thia-

mine directly, and it may be assumed that in the above-observed TDP synthesizing reactions the final steps are the common pathway, namely TDP is synthesized *via* ATP-reaction ultimately. In this experiment the reaction product, TDP, was detected only through its coenzymic activity in the eluate from the paper. To get the reaction product materially, PEP was used as ATP-regenerator to enhance the yield of the product. As is illustrated in Figs. 7 and 8, chromatographic analyses showed the main product to be TDP as the enzymatically active substance in the experimental range. From these chromatographic patterns TMP or thiaminetriphosphate was not detected as the intermediary product.

Enzymic Composition of the System for TDP Formation—According to the above deduction, the enzyme which transfers phosphate from ATP and the enzyme which produces ATP should be different. An attempt was made to separate these enzymes from each other as described in the section under Method. Fig. 9 shows the relative activity of these reactions in each purifi-

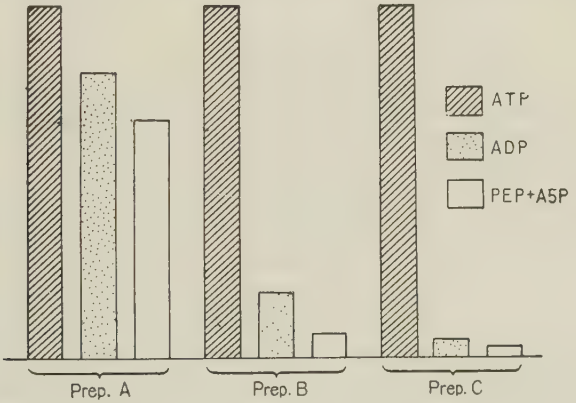

FIG. 9. Comparison of the activity of the three typical reactions at each step of the purification. Values are presented in percentage assuming the activity of ATP as 100 at each step of the purification. Preparation A, B, and C are the same as those described under the Method.

cation step. According to this result, the ratio of the activity of ADP- or PEP+A5P-reaction to that of ATP became lower with the progress of the purification, and at last a preparation was obtained which solely contained the enzyme for ATP-reaction and was practically free from other activities. This fact indicates conclusively that the principal reaction is the ATP-reaction, and the other reactions are accompanied as a secondary mechanism.

By use of the enzyme preparation C described in the Method, it is possible to observe the effect of ADP on the ATP-reaction in a pure state, because the preparation has no activity of adenylate kinase. Thus, an experiment was made to know the proper effect of ADP on ATP-reaction using an ADP preparation which did not contain A5P. As shown in Table II, ADP

showed an inhibitory effect in a similar order of A5P.

Reversibility of the Reaction—From the above experiments it would be concluded that the reaction T+ATP → TDP+A5P is the only direct reaction for TDP formation under physiological condition. Thus the possibility for the reversibility of the reaction starting from TDP and A5P each in concentration of $5\times10^{-3}\,M$ was examined. The result indicated that there is no or extremely little possibility of the reversal reaction because of no appearance of detectable ATP. However, production of ATP in trace amount cannot be denied on account of the limitation of the determination system.

Effect of Inhibitor—The enzyme is characterized by insensitivity to *p*-chloromercuribenzoate* at $10^{-4}\,M$, NaF at $10^{-2}\,M$, arsenite at $10^{-2}\,M$, and iodoacetate at $10^{-2}\,M$, but with ethylenediamine tetraacetate it was marked inhibited: 26, 71, and 100 per cent inhibition was observed at $10^{-3}\,M$, $3.3\times10^{-3}\,M$, and $10^{-2}\,M$, respectively. Inhibitory effect of metallic ions, some thiamine derivatives, and nucleotides has been partly described already and others are reserved for a later paper.

DISCUSSION

Upon considering the above experiments, it may be concluded that the enzymatic constitution for TDP synthesis in rat liver preparation is composed of the enzymes for ATP-reaction as a kernel and other accessory ATP-producing reactions, such as adenylate kinase and pyruvate kinase for the system of ADP and A5P+PEP, respectively. Thus the mechanism of biosynthesis of TDP could be reasonably assumed to occur in one-step transpyrophosphorylation. For further elucidation of this mechanism, the establishment of the complete balance sheet is required. However, this reaction has a number of disadvantageous conditions for the accomplishment, for instance, sluggish nature of the reaction causing intervention of other factors. On account of these difficulties the demonstration of the complete stoichiometric relationship was not successful, although the reasonable formation of TDP and A5P was sometimes observed in the ATP-reaction. Some workers reported the participation of inorganic phosphate, *i.e.* orthophosphate (*5*) and pyrophosphate (*6*), to the reaction but these were all negative in the present experiment. In the progress of the reaction thiaminetriphosphate and monophosphate were suggested by Greiling (*21*) as a possible intermediate in yeast preparation, but neither of them was found in the present system at any stage of the reaction. Detailed mechanism of the transpyrophosphorylation if it takes place, namely the direction of the attachment, could not be elucidate from the present experiment, and the final conclusion must await further investigation. Several years ago, it was reported that the phosphate(s) of TDP is incorporated into the labile phosphates of ATP (*22*, *23*), and a speculation was presented that TDP might act as an energy transmitter in oxidative

* The values were calculated by subtracting the effect caused from the determination system, because of the nature of SH-enzyme of apocarboxylase.

decarboxylation. Negative result for the backward reaction in the present experiment might be cause from the use of soluble enzyme in contrast to the use of "particulate fraction" in the former experiments which handled by sensitive radiochemical analysis.

SUMMARY

Enzymatic synthesis of cocarboxylase in rat liver preparation was studied. For this reaction the enzyme requires thiamine as a phosphate acceptor, ATP as a phosphate donor, and divalent cation(s), Mg^{++} or Mn^{++}, as an activator. Among the adenine nucleotides tested only ATP could serve as a direct substrate and other nucleotides, such as ADP and A5P, did not act as a phosphate donor but behaved as an inhibitor. TMP did not act as a phosphate acceptor but behaved also as an inhibitor. The effectiveness of ADP and A5P+PEP in the crude system may be interpreted as the result of intermediary formation of ATP brought about by other enzymes. Thus, it could be concluded that the only possible mechanism for the reaction is a one-step transpyrophosphorylation. The reaction could not be reversed easily starting from TDP and A5P. The Michaelis constants for thiamine and ATP were calculated as $4.5\times10^{-7}\,M$ and $8.3\times10^{-4}\,M$, respectively. The enzyme was inhibited by ethylenediamine tetraacetate.

The author express his sincere thanks to Prof. N. Shimazono for guidance and encouragement during the course of this investigation.

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STUDIES ON THE RIBONUCLEOPROTEIN PARTICLES

VI. EFFECT OF UREA ON THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES ISOLATED FROM RAT LIVER

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Recently Elson (*1*, *2*) has reported that the ribonucleoprotein (RNP) isolated from *Escherichia coli* is dissociated to RNA and protein by urea treatment, and that urea also activates the latent ribonuclease (RNase) of the RNP remarkably. From these findings he has suggested that the RNP contains RNA and proteins linked together by hydrogen bonds and disruption of these hydrogen bonds would initiate the RNase activity of the RNP.

The RNP isolated from rat liver microsomes was also found to contain RNase (*3*, *4*), and to be dissociated to RNA and protein in strong saline or in alkaline media (*5*). It seems to be interesting, therefore, to examine the effect of urea on the dissociation, or the RNase activity of the RNP. The present paper is concerned principally with the effects of urea on the microsomal RNP of rat liver, its dissociation effect having been examined by electrophoretic, centrifugal, and paperchromatographic methods, while its activation of RNase by incubating the RNP with yeast RNA in urea solution.

EXPERIMENTALS

Isolation of the RNP—The RNP was isolated from liver microsomes of adult rat by sodium deoxycholate treatment as reported previously (*6*) and dissolved in the Miller-Golder's buffer (*7*) of ionic strength 0.1 and pH 7.0.

Chemicals—Analytical grade urea was dissolved in the Miller-Golder's buffer of pH 7.0, its concentration being varied from 1.0 to 10.0 M. All the other chemicals used were also analytical grade.

A. Physicochemical Studies

Electrophoresis: All the electrophoretic analysis were carried out in buffered solution of urea, because removal of urea by dialysis may lead to ambiguity in determining the effects of urea. To 0.6 ml. of the RNP solution of which concentration was about 20 mg. per ml., 0.6 ml. of the buffered solution of urea was added in the cold at a final concentration of 2.0, 4.0 and 6.0 *M* respectively, and these solutions were analysed by an electrophoretic apparatus in a corresponding buffered urea solution without dialysis or after dialysis against the same solutions for 2 or 24 hours. Hitachi Model HTD-1 Eelectophoretic Apparatus was exclusively used and electrophoresis was carried out at 4°.

After electrophoresis samples were pipetted out of the cell to determine RNA and protein content by the modified Schmidt-Thannhauser's method (*6*). Conductivity of the buffered solution of urea was determined by a Wheatstone bridge at 0° and the mobility at 0° was calculated from the distance of migration.

Centrifugal Studies: To 1.0 ml. of the RNP solution, 7.0 ml. of buffered urea was added at a final concentration of 1.0–6.0 *M* respectively and after standing 24 hours in the cold, these solutions were centrifuged at 7,500×*g* for 15 minutes, and then the supernatants were further centrifuged at 105,000×*g* for 120 minutes by a Hitachi Model 40 P ultracentrifuge, dead space in each of the centrifuge tubes being filled by liquid paraffine as previously described (*5*). After centrifugation, RNA and protein in all the pellets and supernatants were determined by the same method stated above (*6*).

Paperchromatography: Twenty μl. of the RNP solution of which concentration was about 20 mg. per ml. was applied in a narrow band about 5 cm. from one end of a strip of Toyo No. 50 paper, 35×2 cm., which was hanged in a tank containing a 1–2 cm. layer of the cold urea solution. The buffered urea solution (0–6 *M*) was allowed to ascend for several hours in the cold (about 5°). After removal from the tank, the papers were dried in a current of warm air from a hair drier, care being taken to avoid a rise in temperature at the paper surface. After drying, RNA was located in ultraviolet light at 253.7 mμ, either visually, or photographically, and protein was located by staining with 0.05 per cent bromphenol blue (*8*).

For semiquantitative analysis, papers were cut out 3 cm. in width from the spots located in ultraviolet light, then eluted and hydrolysed with 2.0 ml. of *N* NaOH for 1–2 hours at 37°. RNA content of the samples was determined from the optical density at 260 mμ, in a Hitachi Model HPU-2 Spectrophotometer, of the acidified NaOH extract, an extinction coefficient of 34.2 per mg. per ml. per cm. being used for calculation. The protein content in NaOH extract was so small that it was directly colored by the Folin's reagent according to Lowry *et al.* (*9*) or Ramachandran *et al.* (*10*), and optical density at 750 mμ was read. To allow for ultraviolet absorbing and Folin positive substances in the paper, blanks are cut equal in area to the spots and at equal distances from the starting line, and treated as well to read at the same wave lengths as the corresponding spots.

B. Biochemical Studies

Yeast RNA: Yeast RNA generously supplied by Dr. Y. Kuroyuwa of the Kirin Beer Research Institute, Amagasaki, was dissolved in the Miller-Golder's buffer of pH 7.0 before use, its pH being corrected again to pH 7.0 with *N*/10 NaOH after dissolution, and kept in the cold.

Autodegradation of the RNP in the urea solution: To each 1.0 ml. of the RNP solution, 1.0 ml. of the cold buffered urea solution was added at a final concentration of 0 *M* (control), 2.0 *M* or 4.0 *M* respectively, and these solutions were incubated at 37°.

RNase Activity of the RNP in the Urea Solution: To 0.5 ml. of 0.5 per cent yeast RNA solution, 1.0 ml. of the buffered urea solution and 0.5 ml. of the RNP solution were added successively at a final concentration of 0 *M* (control), 2.0 *M* or 4.0 *M* with respect to urea, and incubated at 37°.

Assay Methods: After incubation for a definite interval, RNA in the samples was precipitated either by uranium reagent (uranium method), or by perchloric acid (perchloric acid method). In the former method, an aliquot of uranium reagent (*11*) was added, centrifuged, and the supernatant was diluted ten times by distilled water so as to determine its nucleotide content by reading the optical density at 260 mμ as stated above. As molar extinction coefficient of perchloric acid at 260 mμ is by far less than

that of trichlor acetic acid, uranium acetate was used by dissolving it in *N* perchloric acid in place of 2.5 per cent trichlor acetic acid (*12*). In the latter method, an aliquot of ice cold *N* perchloric acid was added in the cold, and the washed precipitate was incubated with *N* NaOH at 37° for 1 hour. RNA content was also determined from the optical density at 260 mμ of the acidified NaOH extract.

RESULTS AND DISCUSSIONS

A. Physicochemical Studies of the RNA Treated with Urea

(*1*) *Electrophoretic Analysis*—For the purpose of demonstrating the dissociation of the RNP in urea, no other method seems to be so direct as the Tiselius' electrophoretic analysis carried out in the buffered solution of urea, and, if the RNP is dissociated in urea, RNA and protein are expected to migrate independently in an electric field as in the case of the RNP treated with alkali (*5*).

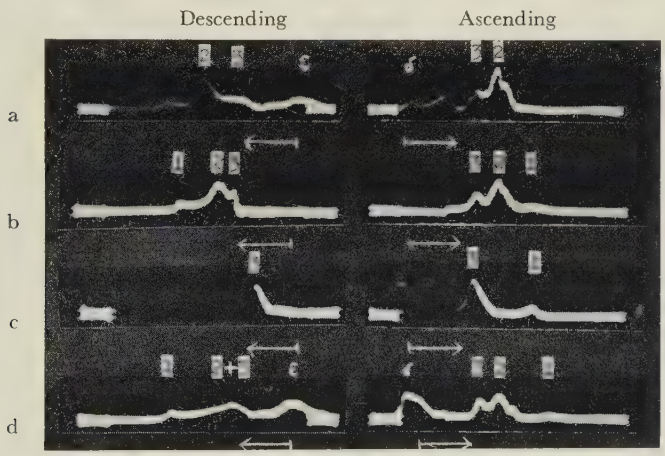

FIG. 1. Electrophoretic patterns of the RNP treated with urea. a: 2 *M* urea, duration of the urea treatment was held to a minimum (about 20 minutes before the start of electrophoresis in this experiment), b: 2 *M* urea, 2 hours, c: 2 *M* urea, 24 hours, d: 4 *M* urea, 2 hours.

Fig. 1-a, b and c show the electrophoretic patterns of the RNP in 2.0 *M* buffered urea solution analysed without dialysis (a), after dialysis for 2 hours (b), or for 24 hours respectively. Judging from its mobility, the Component 2 probably represents the RNP (*13*), which gradually decreased with the duration of urea treatment, while a compensatory increase in the component 3 was observed. A small faster Component (Component 1) also appeared, of which average ascending and descending mobilities were -14.9 and -14.1×10^{-5} cm^2. per sec. per volt respectively. As these values are almost equal to the mobilities of yeast RNA under the same experimental conditions, this component probably represents free RNA released out of the RNP. Prolonged

dialysis in urea resulted in the complete transformation of the Component 2 to 3 and the turbidity of the solution also increased as seen in Fig. 1-c, suggesting that the turbidity increase is related with the appearance of the Component 3.

The degradation of the RNP in more concentrated urea such as 4.0 or 6.0 *M* being similar with that in 2.0 *M* as long as it was observed by electrophoretic method, only the electrophoretic patterns of the RNP treated with 4.0 *M* urea for 2 hours is illustrated (Fig. 1-d).

In these patterns, it is noted that the amount of RNA released out of the RNP by urea is too small to conclude that it was completely dissociated to RNA and protein. As chemical analysis also showed that either protein or RNA were hardly degraded by the cold urea treatment, it is estimated that Component 3 would not represent the protein moiety of RNP, but the denatured RNP. This estimation is supported either by the similarity in the electrophoretic patterns of Fig. 1-d with those of the recombined RNP reported previously (*5*), and by the following centrifugal studies.

(*II*) *Centrifugal Studies*—In Fig. 2 are shown the results of a typical

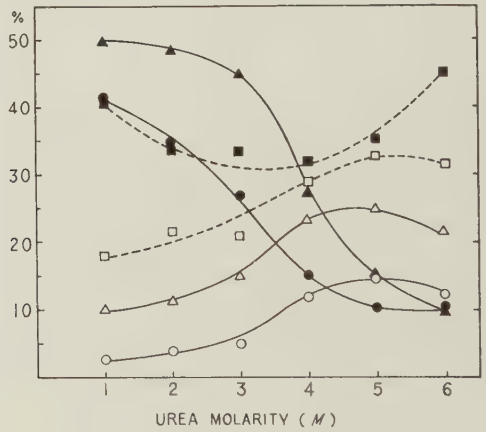

FIG. 2. Centrifugal study of the RNP treated with urea. The RNP was treated with urea for 24 hours, centrifuged at 2,500×*g* for 15 minutes and then the supernatants were further centrifuged at 105,000×*g* for 120 minutes. RNA and protein in all the pellets were determined and the RNA content was calculated.

○ : % recovery of RNA to
△ : % recovery of protein to
□ : RNA content of
} 2,500×*g* sediments

● : % recovery of RNA to
▲ : % recovery of protein to
■ : RNA content of
} 105,000×*g* sediments

experiment, where, it is noted that the RNA contents in all the pellets of

2,500×g and 105,000×g were always about 20–30 per cent and 30–40 per cent respectively. This result does not always agree with the assumption that the RNP is dissociated by urea into RNA and protein moiety, because, if so, the protein, being hardly soluble in neutral buffer (*14*), is expected to be recovered to these pellets as long as it is not solubilized by urea. The previous estimation that Component 3 probably represents denatured RNP is also supported by this finding, because that part of the Component 3 which was responsible for its turbidity in the electrophoretic analysis should have been recovered in the pellets of 2,500×g, of which RNA content was as much as 30 per cent.

(*III*) *Paperchromatography*—Fig. 3-A is the ultraviolet print at 253.7 mμ of the paperchromatogram of the RNP developed in 0 M (control), 2 M, 4 M and 6 M urea respectively. These figures show that the more concentrated urea was used, the more ultraviolet absorbing material is found near solvent front. Fig. 3-B is the corresponding figures of the paperchromatagram of the RNP stained with bromphenol blue. Comparing A with B, it may be concluded that the paperchromatography succeeded to demonstrate almost complete dissociation of the RNP. Elution experiment (Table I), however, gives a somewhat contradictory result that the ultraviolet absorbing spot found

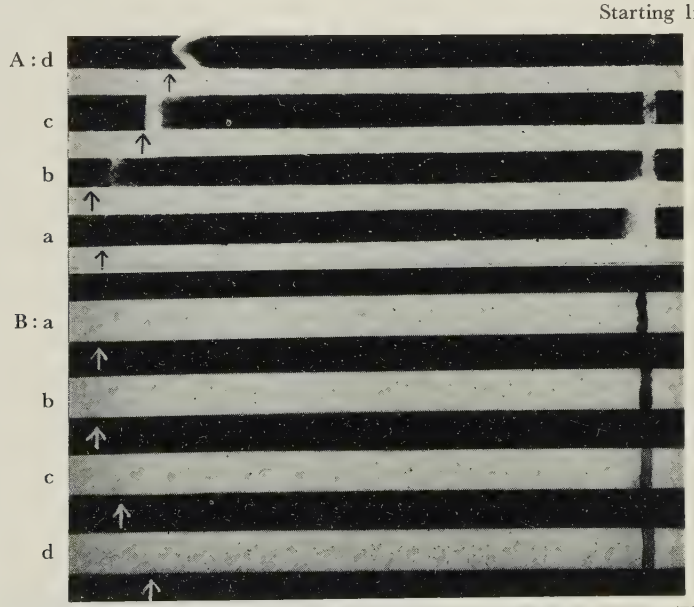

FIG. 3. Ultraviolet prints (A) and bromphenol blue staining (B) of the paperchromatograms of the RNP developed in 0 M (a : control), 2 M (b), 4 M (c), 6 M (d) urea solution. Arrows show the position of solvent front.

near solvent front contains a considerable amount of Folin positive substances. The reason why such a discrepancy appeared was not examined

further, however, the staining with bromphenol blue seems to depend much on the electrochemical properties and on the molecular weight of the protein. It is possible, therefore, that the Folin positive substances in the ultraviolet absorbing spots had hardly any affinity to bromphenol blue because of their electrochemical properties or they may be RNA-bound-amino acid or -polypeptides. Otherwise they might be lost during the staining procedures.

TABLE I

Determination of RNA and Protein in the Eluted Solutions from the Paperchromatogram of the RNP Developed in Urea Solution

(Avarage values of two experiments).

Concentration of urea		0 *M*	2 *M*	4 *M*	6 *M*
Starting line	RNA ($\mu g.$)	52	37	18	16
	Protein ($\mu g.$)	80	68	40	5
Ultraviolet absorbing spot near the front	RNA ($\mu g.$)	0	22	68	77
	Protein ($\mu g.$)	0	0	15	20

It has been reported that the RNP is dissociated by strong saline and by alkali so that electrostatic force is also important for the bonding between RNA and protein of the RNP (*5*). The results obtained in this report, therefore, seem to be tentatively interpreted as follows: Strong urea solution probably disrupted not only the intramolecular hydrogen bonds of RNA and protein respectively, but also the intermolecular hydrogen bonds between RNA and protein of the RNP. At pH of 7.0, however, ionic bonds are still effective in linking the two components together. The electrophoretic and centrifugal analysis, therefore, have failed to demonstrate the dissociation of the RNP as it can migrate together both in an electrical and in a gravitational field. Paperchromatography in urea, on the contrary, seems to succeed to demonstrate the dissociation of the RNP, probably because protein might have much more affinity to paper than RNA. Various molecular kinetic data should be accumulated before one can clearly understand the mechanism of the effect of urea on the RNP.

B. Biochemical Studies.

(*1*) *Effect of Urea on the Precipitation of RNA by Uranium Reagent and Perchloric Acid* -In the study of autodegradation or of RNase activity of the RNP in urea solution, it is necessary to examine previously the effect of urea on the precipitation of RNA by uranium reagent or by perchloric acid. Ten mg. of yeast RNA was dissolved in 1 ml. of *N* NaOH and incubated at 37° for 25-120 minutes. After incubation, 1 ml. of urea solution was added and then RNA was precipitated either by 2 ml. of uranium reagent or by *N*

perchloric acid. After centrifugation, nucleotide in the supernatant was determined by ultraviolet absorption.

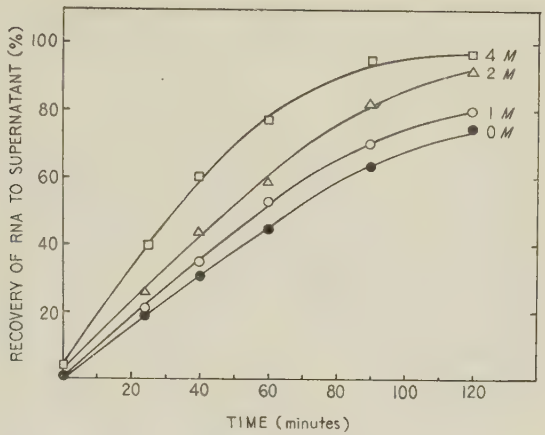

FIG. 4. Effect of urea on the precipitation of RNA by uranium reagent. Ten mg. of yeast RNA dissolved in 1 ml. of *N* NaOH was incubated at 37°. After incubation, various concentration of urea (1 ml.) and uranium reagent (2 ml.) was successively added, and per cent recovery of RNA to supernatant was determined.

Fig. 4 shows that the precipitability of RNA by uranium reagent depends much on the concentration of urea. Similar relation was obtained when cold 0.5 *N* perchloric acid was used instead of uranium reagent. It is suggested that the reasonable interpretation of the experimental results always necessitate to calibrate the results with a suitable control.

(*II*) *Effect of Urea on the Autodegradation of the RNP*—Fig. 5 shows the effect of urea on the autodegradation of RNA of the RNP. As a control, 1.0 ml. of the RNP was incubated for 5–20 minutes at 37° without adding urea, and after incubation, a mixture of 1.0 ml. uranium reagent and 1.0 ml. urea was added so as to make the final concentration of urea 2.0 *M* (▲) and 4.0 *M* (■) respectively. Under such conditions, the difference between △ and ▲, or □ and ■ indicates the accelerated degradation of the RNP by urea, and so it is apparent that the autodegradation of RNA was somewhat accerelated by urea. Similar results were obtained when perchloric acid was used instead of uranium reagent (Fig. 6). The latter method also showed that the degradation of protein of the RNP was hardly initiated by urea (see *ref.* (*4*)).

(*III*) *Effect of Urea on the RNase Activity of the RNP*—The effect of urea on the RNase activity of the RNP was examined either by the uranium method (Fig. 7) or by perchloric acid method (Fig. 8). In these figures the broken lines represent control experiments where urea was added at the end of the incubation as stated in the previous section. The uranium method clearly demonstrated the acceleration of the RNase activity by urea, while

the activation was so slight that the perchloric acid method failed to show the activation.

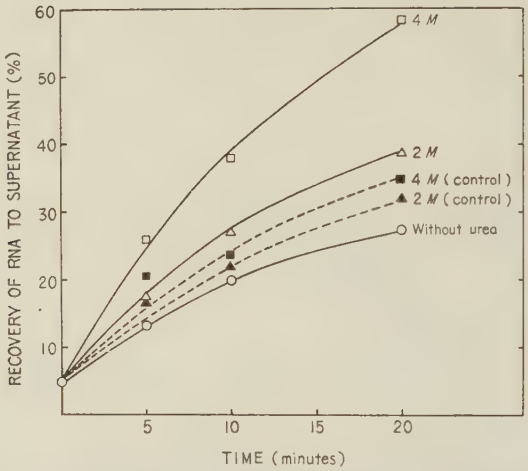

FIG. 5. Spontaneous degradation of RNA of the RNP in urea solution (uranium method). The RNP was incubated at 37° without urea (○), with 2 *M* (△), or 4 *M* (□) urea and RNA was precipitated with uranium reagent. (▲) and (■) represents control experiment, where urea was added after incubation at the final concentration of 2 and 4 *M* respectively.

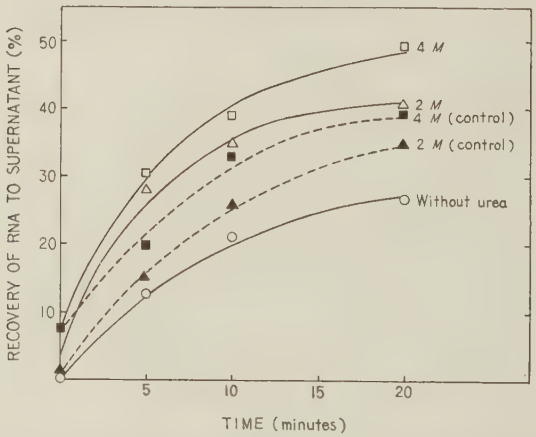

FIG. 6. Spontaneous degradation of RNA of the RNP in urea solution (perchloric acid method). The RNP was incubated in the similar manner as in Fig. 5, and after incubation, RNA was precipitated with *N* perchloric acid. Meaning of the signs in this figure is as same as in Fig. 5.

The protease activity of the RNP was also determined by the Kunitz's

method (*15*) in the presence of urea, and it was found that it's activity was hardly initiated by urea.

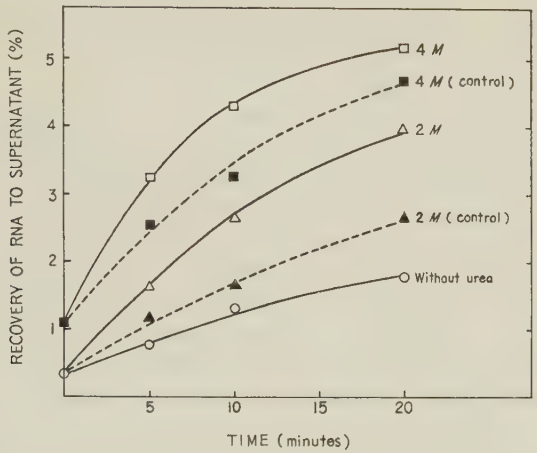

FIG. 7. RNase activity of the RNP in urea solution (uranium method). Yeast RNA (0.5 per cent 0.5 ml.) and the RNP solution were incubated at 37° without urea (○), with 2.0 *M* urea (△) or with 4.0 *M* urea (□) and then RNA was precipitated with uranium reagent. In control experiments, urea was added after incubation.

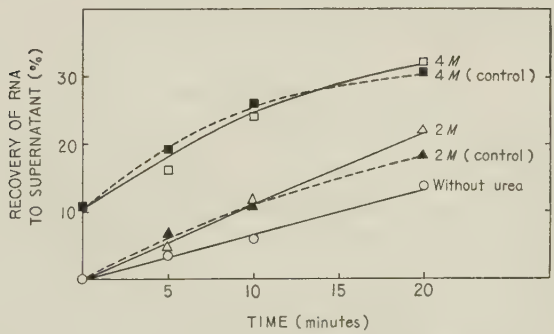

FIG. 8. RNase activity of RNP in urea solution (perchloric acid method). Yeast RNA and the RNP was incubated in the similar manners as in Fig. 7 and after incubation RNA was precipitated with *N* perchloric acid. Meaning of the signs in this figure is as same as in Fig. 7.

From these experiments, it may be concluded that the autodegradation of RNA and RNase activity of the RNP are slightly activated by urea. This conclusion seems to be in good agreement with the previous one that the RNP isolated from the liver microsomes of adult rat probably contains almost all the RNase in an active state (*4*).

SUMMARY

The RNP was isolated from the liver microsomes of adult rat and the effect of urea on the dissociation and on the RNase activity of the RNP were examined.

1. Electrophoretic analysis showed that the RNP was hardly dissociated to free RNA and protein at pH 7.0 even in the concentrated urea solution, though it was gradually degraded to a slower component (component 3), of which RNA and protein are probably linked loosely by ionic bonds. This estimation is also supported by centrifugal analysis.

2. Paperchromatography in urea succeeded to demonstrate the dissociation of the RNP. When the RNP was developed in the concentrated urea solution, protein stained with bromphenol blue was always found at or near the starting line, while most RNA was detected near the solvent front. Eluted solutions from the paper, however, showed that this RNA contained considerable amount of Folin positive substances.

3. Either the autodegradation of the RNP and the RNase activity of the RNP were slightly activated by urea.

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INHIBITION OF YEAST GLYCYLGLYCINE DIPEPTIDASE BY AMINO ACIDS

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During the course of the previous study (*1*) on yeast glycylglycine dipeptidase, it was discovered that the enzyme activity was significantly enhanced on dialyzing the crude autolysate prepared from yeast. As a result of the following examinations, it was found that this behavior of the enzyme is to be accounted for, at least partly, by the presence of various amino acids in the crude autolysate and their removal through dialysis. The inhibitory effect of amino acids upon dipeptidase has been known since the work of Grassmann *et al.* (*2*), who reported that the dipeptidase activities of yeast and kidney extracts towards alanylglycine, glycylglycine, leucylglycine were strongly inhibited by the presence of various amino acids. Also in the present case, the addition of various amino acids to the partially purified yeast enzyme was found to cause a more or less marked retardation of the reaction rate. The inhibiting effect of each individual amino acid was examined under various experimental conditions. The results obtained are briefly described in the followings.

MATERIALS AND METHODS

Yeast glycylglycine dipeptidase was prepared from baker's yeast as described previously (*1*). Prior to the addition of substrate, the enzyme was preincubated with amino acid in the presence of $CoCl_2$ (0.001 *M*) for 10 minutes at 35°. The solution was buffered with 0.05 *M* veronal (pH 7.8). The activity of the enzyme was determined by measuring the hydrolysis of the substrate, glycylglycine, by the formol titration method. The typical reaction mixture consisted of 0.05 *M* glycylglycine, 0.001 *M* $CoCl_2$, 0.05 *M* veronal buffer (pH 7.8) and the enzyme preincubated with inhibitor as described above. The reactions were run at 35°. The final concentration of enzyme was around 10 γ protein nitrogen per ml. of reaction mixture.

RESULTS AND DISCUSSION

As a result of preliminary experiments, L-leucine was found to be most effective, among the amino acids tested, in inhibiting the action of yeast glycylglycine dipeptidase. Detailed kinetic studies, therefore, were mostly

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performed with this amino acid as inhibitor. Fig. 1 shows the inhibitory

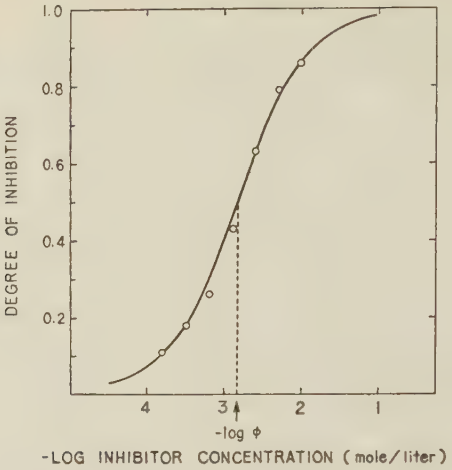

FIG. 1. Inhibition of glycylglycine dipeptidase by L-leucine. Experiments were performed at 35° and pH 7.8 in the presence of 0.05 *M* glycylglycine, 0.001 *M* $CoCl_2$ and L-leucine. Degree of inhibition was expressed as described in the text.

effect of L-leucine on the enzyme activity. The degree of inhibition is defined as follows:

$$H = 1 - \frac{V_i}{V_o} \qquad (1)$$

where V_i and V_0 stand, respectively, for the reaction rates in the presence and absence of the inhibitor. The degree of inhibition as plotted against the logarithms of concentration of L-leucine, gives a sigmoid curve of first order, which is represented by the following equation:

$$H = \frac{[I]}{[I] + \phi} \qquad (2)$$

where [I] stand for the concentration of inhibitor (I); ϕ is a constant numerically equal to the concentration of inhibitor producing 50 per cent inhibition of the reaction. The values for ϕ were found to vary when the substrate concentration was varied in the tests for the inhibition under investigation. The results of this series of experiments are presented in Fig. 2. It will be seen from this figure that the increase in concentration of substrate results in an increase in the value of ϕ, indicating that the inhibiting action of L-leucine is weakened by the presence of substrate, *i.e.*, the substrate glycylglycine, and the inhibitor L-leucine, are mutually competitive with respect to their action towards the enzyme.

The inhibition of the reaction was also studied as influenced by varied concentration of activator (Co^{++}). The results are presented in Fig. 3-a and

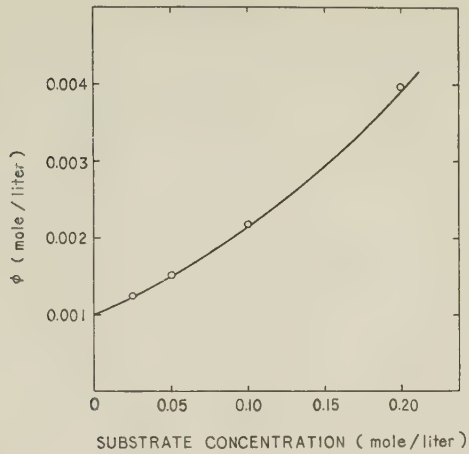

FIG. 2. Values for ϕ as a function of substrate concentration. ϕ values were obtained for various substrate concentrations in a manner described in the text. Points plotted were obtained experimentally and solid curve was calculated according to Eq. 6.

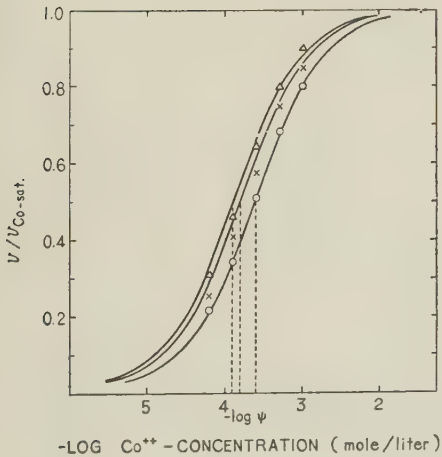

FIG. 3-a. Activity of glycylglycine dipeptidase as a function of Co^{++} concentration at various inhibitor (L-leucine) levels. Points plotted represent the experimental values. Solid lines were drawn according to Eq. 7. (○) without inhibitor; (×) 5×10^{-4} *M* leucine; (△) 10^{-3} *M* leucine.

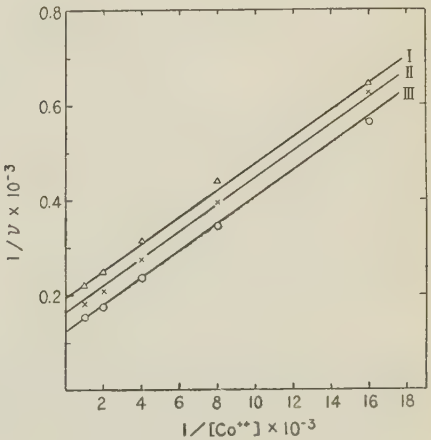

FIG. 3-b. Reciprocal reaction velocity at various levels of inhibitor (L-leucine) as a function of reciprocal concentration of Co^{++}. I, 10^{-3} *M* leucine; II, 5×10^{-4} *M* leucine; III, without inhibitor.

b. It will be seen from Fig. 3-a that the apparent affinity of Co^{++} ion towards the enzyme increases with increasing concentration of inhibitor. By replotting these data in terms of $1/v$ against $1/[Co^{++}]$ (Fig. 3-b), a set of

parallel lines are obtained; I and II correspond to two fixed concentrations of inhibitor and III for the absence of inhibitor. Actually the curves in the previous figure (Fig. 3-a) are drawn using the values for the Co^{++}-saturated velocity ($V_{Co-sat.}$) for each inhibitor concentration, as computed from the intersects of the lines with the vertical axis in Fig. 3-b. These results suggest that the inhibitor is non-competitive with the activator. This will be proved in later section.

In the previous work (*1*) dealing with the same enzyme, the present author has proposed the following scheme for the enzyme reaction*:

$$\begin{array}{l} E+A \overset{K_1}{\rightleftharpoons} EA, \quad EA+S \overset{K_2}{\diagdown\!\!\diagup} \\ \qquad\qquad\qquad\qquad\qquad\quad EAS \xrightarrow{k} \text{product} \\ A+S \overset{K_3}{\rightleftharpoons} AS, \quad E+AS \overset{K_4}{\diagdown\!\!\diagup} \\ E+S \overset{K_5}{\rightleftharpoons} ES \text{ (inactive complex)} \end{array}$$

In the above set of formulae, K_1, K_2, K_3, K_4 and K_5 represent the dissociation constants for the reaction steps indicated. The experimental results presented above are also best accounted for by the above set of formulae, if one additional assumption is made pertaining to the step of combination of enzyme with inhibitor:

$$EA+I \overset{K_i}{\rightleftharpoons} EAI \tag{4}$$

$$K_i = \frac{[EA][I]}{[EAI]}$$

According to the above stated reaction scheme, the rate of reaction V_i in the presence of inhibitor is represented by the following equations:

$$V_i = \frac{k[e]}{1+\frac{K_2}{[S]}+\frac{K_1K_2}{[S][A]}+\frac{K_1K_2}{K_3[A]}+\frac{K_1K_2}{K_5[A]}+\frac{K_1K_2[S]}{K_3K_5[A]}+\frac{K_2[I]}{K_i[S]}} \tag{5}$$

$$\phi = K_i\left(1+\frac{K_1}{[A]}+\frac{[S]}{K_2}+\frac{K_1[S]}{K_3[A]}+\frac{K_1[S]}{K_5[A]}+\frac{K_1[S]^2}{K_3K_5[A]}\right) \tag{6}$$

Using the values of ϕ observed in Fig. 1 and the values for K_1, K_2, K_3, K_4 and K_5 which were obtained previously under similar experimental conditions, the value for K_i was computed to be as follows:

$$K_i = 10^{-3} \text{ mole/liter}$$

The changes in values of ϕ as the results of varying the substrate concentration are also in satisfactory harmony with the theory. In Fig. 2 the curve in solid line was drawn according to Eq. 6. The fitness of experimental values with the theoretical curve is satisfactory.

* In the complete reaction scheme developed in the previous paper, an assumption has been made concerning the formation of an enzyme-activator complex of the second order (EAA). This point of the scheme can, however, safely be neglected in the present case, in which only lower concentrations of activator are dealt with.

The results of the third series of experiments analyzing the reaction rate (in the presence or absence of inhibitor) as a function of Co^{++} concentration are again in accord with the theory. The curves in Fig. 3-b are drawn according to the theory (Eq. 7), using the above obtained value for K_i:

$$\frac{V}{V_{Co-sat.}} = \frac{[A]}{[A]+\phi} \qquad (7)$$

$$\phi = \frac{K_1 K_2}{K_3 K_5} \frac{([S]+K_5)([S]+K_3)}{[S]+K_2\left(1+\frac{[I]}{K_i}\right)}$$

Inhibition by other amino acids was also investigated with the purpose of inquiring the relationship between inhibitory action and chemical structure of amino acids. The value for the inhibition constant (ϕ) was estimated as the concentration of each amino acid producing 50 per cent inhibition of the enzyme reaction under investigation. The reaction mixture was the same as in the above-described experiments. The data presented in Table I show that the substitution at the amino or carboxyl group results in a decrease in inhibitory action. Thus the benzoyl derivative of leucine was only about one-tenth, and leucinamide about one-sixth as effective as the original amino acid.

TABLE I

Effect of Leucine Derivatives on Yeast Glycylglycine Dipeptidase

Compounds	ϕ (M)
L-Leucine	1.5×10^{-3}
L-Leucinamide	9.0×10^{-3}
Benzoyl-L-leucine	1.5×10^{-2}

TABLE II

Optical Specificity in Inhibition of Yeast Glycylglycine Dipeptidase by Amino Acids

Amino acids	ϕ (M)
L-Leucine	1.5×10^{-3}
DL-Leucine	3.0×10^{-3}
L-Isoleucine	1.8×10^{-3}
DL-Isoleucine	1.1×10^{-2}

Another experiments concerning the optical specificity of the amino acid inhibition were also performed. In these experiments, the ϕ value of the

L-form of each amino acid was compared with that of the DL-form. As shown in Table II, only the L-form of amino acid seems to produce the inhibition of enzyme reaction.

Inhibitory activity of α-amino acids varied significantly with differences in the structure and molecular size of their side chains. In Table III were summarized effects of the length of the carbon chain. In the series of straight chain monoamino-monocarboxylic acids, the inhibitory activity steadily increases from glycine (C_2) to norvaline (C_5). Further extension in carbon chain up to norleucine (C_6), however, did not give rise to increase in the inhibitory action. Similar results have been reported in the inhibition of

TABLE III

Effect of Length of Side Chain of Amino Acid on Inhibition of Yeast Glycylglycine Dipeptidase

Amino acids	ϕ (M)
Glycine	>1.0
DL-Alanine	2.5×10^{-1}
DL-α-Aminobutylic acid	3.7×10^{-2}
DL-Norvaline	1.3×10^{-2}
DL-Norleucine	1.3×10^{-2}

TABLE IV

Inhibition of Yeast Glycylglycine Dipeptidase by Various Amino Acids

Amino acids	ϕ (M)
DL-Phenylalanine	2.2×10^{-2}
DL-Valine	2.2×10^{-2}
L-Tryptophane	6.0×10^{-3}
L-Histidine	8.0×10^{-3}
L-Arginine	1.8×10^{-2}
L-Aspartic acid	4.0×10^{-2}
DL-Serine	$> 10^{-1}$
DL-Threonine	$> 10^{-1}$
DL-Methionine	1.0×10^{-2}
L-Proline	$> 10^{-1}$

arginase by amino acid (*3*). In the case of leucine amino-peptidase, on the other hand, it has been shown that the availability of straight chain amino acid amides as substrates regularly increases with the size of the side chain (*4*). All these facts suggest that the chemical structure of the side chain of amino acid or peptide may be an important factor which should be involved in the specific interaction between enzyme and substrate, or inhibitor.

Inhibitory effects of other amino acids are summarized in Table IV. All these amino acids listed in Table I, II, III and IV have a high affinity towards the activator ion, Co^{++} (*5*). Some of them, glycine (reaction product), threonine, serine and proline, however, showed no detectable inhibition of the enzyme reaction. Moreover, no relationship was established between ϕ values and chelating activities of these amino acids. For instance, in the case of aliphatic amino acids, the inhibitory activity increases in the order of glycine, alanine, valine and leucine, whereas the affinity towards Co^{++} ion decreases in the order of glycine, alanine, leucine and valine (*5*). Also in other peptidases, the amino acids produced as a result of enzyme reaction, are usually stronger chelating agents than the substrate. However, no marked inhibition by reaction products has been reported in these cases. In the case of leucine amino-peptidase, Smith and Speckman have explained the fact in termes of the difference in pK values of amino groups in the peptide and the amino acid (*6*). As may be considered from the above described facts, however, this way of explanation is not applicable at least in our present case. The ultimate cause of the apparent independence of the affinities of amino acids towards the enzyme and towards the activator ion still remains unelucidated.

SUMMARY

1. The inhibition of glycylglycine dipeptidase of yeast by various amino acids was investigated kinetically. The inhibition caused by L-leucine was competitive with substrate but not with Co^{++} ion. A scheme for the inhibition produced by amino acid was proposed which was shown to satisfactorily agree with the experimental results.

2. The inhibitory activities of various amino acids were compared in terms of the affinity constants of these substances towards the enzyme.

The author wishes to express his sincere gratitude to Prof. A. Takamiya for his valuable advices in this work.

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THE EFFECT OF EDTA AND ITS ANALOGUES ON GLYCERINATED MUSCLE FIBERS AND MYOSIN ADENOSINETRIPHOSPHATASE*

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A number of compounds have been known to have a relaxing action on glycerinated muscle fibers in the presence of ATP. These compounds might be classified into three groups represented respectively by Salyrgan (Portzehl (*1*), 1952), EDTA (Bozler (*2*), 1954; Watanabe (*3*, *4*), 1955) and acetone (Ebashi and Ebashi (*5*, *6*), 1959).

Among these agents, EDTA is especially noteworthy, because it has, in addition to the relaxing action, various kinds of actions on myosin and myosin-related systems (*7–13*); for example, it strongly activates the ATPase of myosin in the presence of potassium ions of high concentration (*7–9*).

In view of the important role of divalent cations in myosin and myosin-related systems, the chelating action of EDTA is of interest. The experiments described in this paper were conducted mainly to inquire into the correlation between the chelating action of EDTA and its analogues and their biological actions on glycerol-treated muscle fibers and myosin-A ATPase.

EXPERIMENTAL

Preparation of Glycerol-Treated Muscle Fibers for Measuring Contraction—Glycerol-treated psoas muscle fibers of rabbits, preserved for 2 days to 4 weeks in 50 per cent glycerol at −10°, were immersed in 20 per cent glycerol and dissected in this solution for contraction test. After being attached to the lever, fibers were immersed in 0.015 per cent benzalkonium chloride** (*14*) for three minutes to abolish the relaxing activity of granules contained in fibers. All the contraction tests were made in 0.15 *M* KCl or NaCl solution (unless otherwise noted, KCl solution was used) containing 0.01 *M* $MgCl_2$ and 0.02 *M* Tris maleate buffer (pH 6.8 or 6.6). Other procedures were almost the same as described in the previous paper (*15*).

* The following abbreviations were used in this paper: ATP, adenosine triphoshate; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; CyDTA, 1,2-cyclohexanediaminetetraacetic acid; EEDTA, ethyletherdiaminetetraacetic acid; GEDTA, glycoletherdiaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; EDTA-OH, hydroxyethylethylenediaminetriacetic acid, and Tris, tris-(hydroxymethyl)-aminomethane.

** This could be replaced by 0.02 per cent desoxycholate, 0.2 per cent taurocholate, 0.2 per cent glycocholate, 0.1 per cent cholate, 0.005 per cent sulfate ester of higher alcohol, 0.015 per cent alkylbenzene sulfonate or 0.1 per cent alkylpyridinium.

Measurement of Relaxing Activity of Chelating Compounds—As shown in Fig. 1 the shortening of fibers by ATP was stopped mechanically at a certain point corresponding to 12 per cent shortening of initial length. In the presence of some chelating agent fibers shortened by ATP would begin to lengthen after a certain period of time. This period was used in evaluating the relaxing activity.

Preparation of Myosin-A—Myosin-A was prepared by the method of Szent-Györgyi (*16*).

Measurement of Myosin-A ATPase—Each milliliter of reaction mixtures contained 1.0 μ mole of ATP, 0.6 m moles of KCl, 20 μ moles of Tris buffer (pH 8.5), a specified amount of myosin-A and a specified amount of agent to be tested. Incubation was conducted at 25° for 15 minutes.

EDTA and Its Analogues—obtained from Dojin Pharmaceutical Laboratories (Kumamoto).

RESULTS

Relation between Relaxing Capacity and Chelating Activities of EDTA Analogues—Experiments were conducted using NaCl solution as well as KCl one as the medium to exclude the specific effect of a given monovalent ion.

Fig. 1 shows a part of this experiment; the results are summarized in Table I. No correlation between chelating activity and relaxing activity of

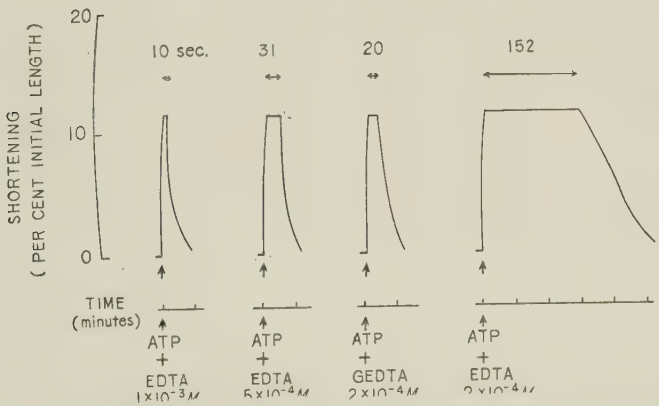

FIG. 1. Relaxing effects of EDTA and GEDTA in the presence of ATP (an illustration of the method evaluating the relative lengthening activities of EDTA analogues).
sec. = seconds. ATP, $5 \times 10^{-3} M$. pH, 6.8. At 22°. For others see the text.

the series of EDTA analogues could be discovered. It is worth noting that GEDTA appeared as a much stronger relaxing agent than EDTA in spite of its much lower chelating capacity at pH 6.8, especially for Mg, whereas Cy-DTA was a weak relaxing agent, notwithstanding its potent chelating action.

Potentiation of Myosin-ATPase by EDTA Analogues—Fig. 2 shows the effects of the various chelating agents on myosin-A ATPase. If we judge the relative values of ATPase-potentiating activities from the concentration of each analogue which produces the same degree of activation, there could not be found

TABLE I

Relaxing Activity, Myosin-A ATPase Potentiating Activity and Pyrophosphate-Action Enhancing Activity of EDTA and Its Analogues

	Chelating activity[1]				Relaxing activity (at pH 6.8)		ATPase[2] potentiating activity (at pH 8.5)	Pyrophosphate enhancing activity (at pH 6.6)
	For Ca		For Mg		in KCl	in NaCl		
	at pH 8.5	at pH 6.8	at pH 8.5	at pH 6.8				
EDTA	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)
CyDTA	0.91	0.93	1.48	1.52	0.10	0.2	2.5	1
GEDTA	4.2	0.15	0.0007	<0.0001	8.2	6.3	<0.01	2
DTPA	0.68	0.029	0.56	0.024	0.12	0.08	0.6	2
EEDTA	0.22	0.015	0.46	0.030	0.37	0.5	0.6	0.5
EDTA-OH	0.007	0.008	—	—	0.25	0.5	0.04	0.4
NTA	0.0002	0.0002	0.002	0.002	0.05	0.04	<0.01	0.5

Activity of each compound was expressed as its value relative to that of EDTA on the basis of molar concentration.

1) Indicate the value of stability constant of each compound relative to that of EDTA calculated from the TABLE VII of the book of Schwarzenbach (*17*) and the TABLE I of the paper of Danzuka and Ueno (*18*).

2) Caluculated from Fig. 2 in this paper.

any relationship between the enzymic activity and the chelating capacity for Ca, as in the case of relaxing activity. In case of Mg, however, a fairly good parallelism was observed between these two activities (Table I.)

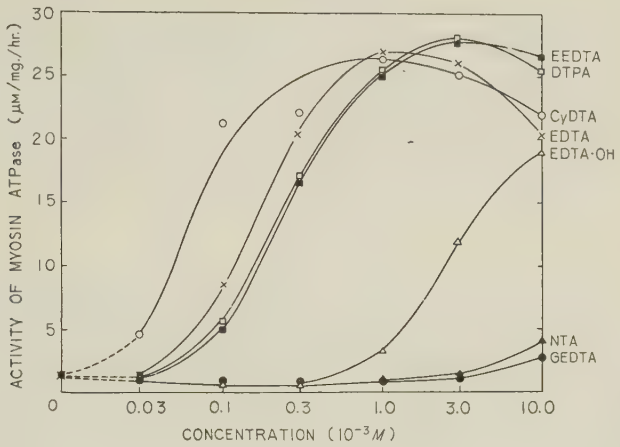

FIG. 2. Acceleration of myosin-A ATPase by EDTA and its analogues.

Ordinate: μM of inorganic phosphate split from ATP per mg. of myosin-A per hour. Abscissa: concentrations of chelating agents.

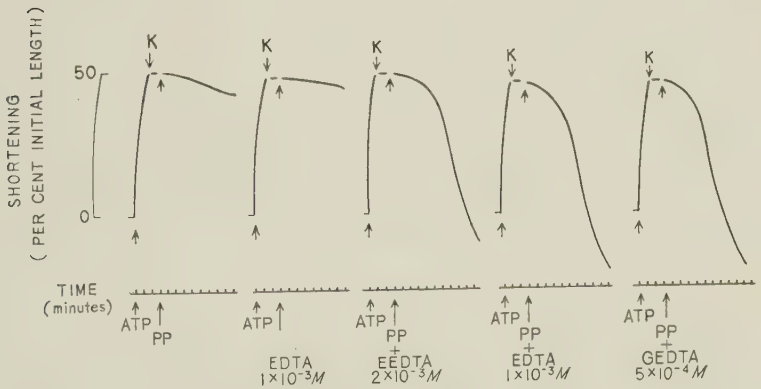

FIG. 3. Acceleration of lengthening action of inorganic pyrophosphate on glycerol-treated fibers by chelating compounds.

PP: inorganic pyrophosphate, $1\times10^{-3}M$. ATP: $5\times10^{-3}M$ ATP. pH: 6.6. K: potassium chloride solution. (See Experimental)

It must be pointed out that among those compounds which showed a saturating degree of ATPase activation in the concentration range used, the activities of saturation level were the same for all compounds.

Enhancement of Plasticizing Action of Inorganic Polyorthophosphate by EDTA

and Its Analogues—It has been shown that acetone intensifies remarkably the plasticizing action of inorganic pyrophosphate and tripolyphosphate (*6*). This is in accord with the observation of Morita and Tonomura* that acetone remarkably strengthened the binding force between pyrophosphate and actomyosin.

These findings suggested a similar experiments with EDTA and its analogues and revealed that these compounds had an action resembling acetone (Fig. 3). In the case of the chelating agents, however, the contractility of fibers was lost after treatment. In addition to this, relative values of plasticity-enhancing activity were different quite sharply from those of relaxing action (Table I).

DISCUSSION

The present paper has revealed that three different kinds of actions of EDTA on myosin-systems, *viz.*, the relaxing action on glycerinated muscle fibers in the presence of ATP, the enhancing action on myosin-A ATPase, and the synergistic action with inorganic polyphosphate on the lengthening of glycerinated muscles, do not have any quantitative correlation with each other. This strongly suggests that the various actions of EDTA on myosin-related systems hardly be explained on the basis of only one principle.

Among the various activities of chelating agents tested in this experiment, only the activating effect on myosin ATPase was found to have some correlation with the chelating capacity for Mg. However, as Friess, Morales and Bowen (*9*) have pointed out, this may not necessarily mean that the activation of myosin ATPase by chelating compounds is due to the simple chelation for Mg.

No significant correlation could be observed between the relaxing activity and the chelating capacity of chelating compounds. It is interesting, however, that GEDTA, which has practically no chelating on Mg. at pH 6.8, in spite of its definite chelating capacity for Ca, shows a remarkable relaxing activity, several times that of EDTA. This suggests that the role of Ca in the relaxation should be further investigated.

SUMMARY

EDTA and six analogues were tested for their relaxing action on glycerinated psoas muscle fibers, their potentiating effect on myosin ATPase and their synergistic action with inorganic polyorthophosphate on glycerinated fibers.

1. The relaxing action of the chelating compounds was shown to have any quantitative correlation with their chelating action on neither Ca nor Mg. GEDTA, which has practically no chelating capacity for Mg under the experimental conditions, showed a remarkable relaxing effect, several times as strong as EDTA.

* Personal communication

2. The potentiating action of the chelating agents on myosin-A ATPase has no correlation with their chelating effect on Ca, but showed some correlation with their chelating capacity for Mg.

3. The lengthening of glycerinated muscle fibers by inorganic polyorthophosphate was markedly accelerated by EDTA and its analogues; thus elongated fibers were found without contractility. This action did not parallel the chelating activity.

4. The abovementioned three actions of chelating agents showed no correlation with one other.

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METABOLISM OF PARA-AMINOBENZOIC ACID

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Many researches have been made on the acetylation and glucuronic conjugation of *p*-aminobenzoic acid (PABA) *in vivo*, but only the counter current distribution method has been employed in the systematic survey of its metabolic products. By this method, Tabor and co-workers (*1*) revealed that PABA was conjugated with glucuronic acid, acetic acid, and glycine in man. Consequently six substances were detected as its metabolites in human urine. Later, in our country, Umeda (*2*) made a research on metabolites of PABA in rabbit urine using the same procedure.

The present authors have attempted to investigate to carry out the metabolic fate of PABA in several kinds of mammals by means of paper-chromatography. And in this report discussions were also made on *N*-glucosides of the metabolites found in rabbit urine after simultaneous administration of PABA and glucose.

EXPERIMENTALS

Materials—PABA, *p*-aminohippuric acid (PAHA) and *p*-*N*-acetylaminohippuric acid (AcPAHA) used in this work were commercial products (Daiichi Kagaku Co.) with no further purification. *p*-*N*-Acetylaminobenzoic acid (AcPABA) (m.p. 250-252°) was prepared according to Kaiser (*3*) and recrystallized from aqueous ethanol. *p*-Aminobenzoyl glucuronide (PABAG) was obtained by the method of Bray *et al.* (*4*) from dog urine after feeding PABA. Its chemical properties were previously reported by one of the authors (*5*). *p*-Aminobenzoic acid-*N*-glucoside (m.p. 125° with decomp.) and *p*-aminohippuric acid-*N*-glucoside were prepared by the method of Hanaoka (*6*). Owing to unstable character of the latter, even in low temperature, we failed to obtain it in crystal form, so the reaction product itself was used for the determination of R_f values on paper-chromatograms.

Human beings and dogs were fed with mixed diet, rabbits with bean-curd leavings, and mice with pressed barley. A dose of 1 g. of PABA was administered orally to man, and one tenth to three tenth grams of PABA per kg. body weight was administered to other animals either orally, intravenously or intraperitoneally. The urine excreted within 3 to 4 hours was collected after the intravenous injection. To collect rabbit urine from ureter, a fine vinyl tube (1 mm. diameter) was inserted into the incized ureter in the op-

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posite direction of urine flow and was fastened with thread. The urine was collected successively from the other end of the tube. In the simultaneous administration of glucose and PABA, the mixture of 15 ml. of 20 per cent glucose and 5 ml. of neutral water solution of PABA was injected to the rabbit intravenously.

The urine was added to about 2 volumes of ethanol and the clear supernatant was used for paper-chromatography. When higher concentration was required, the original urine was concentrated to one tenth volume under reduced pressure below 40°.

Paper-chromatography—The desalted urine was applied on Toyo filter paper No. 50 or No. 51 and the chromatograms were run in methanol-benzene-*n*-butanol-water (2 : 1 : 1 : 1) for about 10 hours and then in *n*-butanol-acetic acid-water (4 : 1 : 5) for about 20 hours. For the localization of spots either Bratton-Marshall's reagent or Ehrlich's reagent was used by nearly the same procedure reported by Nakao (*7*).

The following three sprays were used for the detection of glucose :

(*a*) *Benzidine Reagent* : To 80 ml. of ethanol was added 0.5 g. of benzidine, and mixed with 20 ml. of acetic acid. The chromatograms were sprayed with sufficient amount of this solution and heated for about 30 minutes at 110°.

(*b*) *TTC Reagent*—A solution of 2 per cent triphenyltetrazolium chloride was mixed with equal part of 1 *N* sodium hydroxide just before use. After spraying this reagent the chromatograms were warmed at 40° for about 30 minutes in dark place.

(*c*) *Glucose Oxidase Reagent* : This reagent was freshly prepared by extracting from about 30 cm. of commercial Tes-tape (Eli Lilly Co.) with 5 ml. of water.

Qualitative and Quantitative Analysis—Tes-tape and Clinistix (Ames Co.) were used for detection of glucose, and naphthoresorcin method (*8*) was used for glucuronic acid.

The determination of amines was done by Bratton-Marshall's method (*9*) modified by Kalant (*10*) using Hitachi photoelectric colorimeter with No. 55 filter for PABA and PAHA and No. 53 for PABAG. Being obtained approximately the same absorbance from each amine, filter No. 50 was used for the estimation of total amines.

RESULTS

Metabolic Products of PABA—Six metabolites originated from PABA were detected by two-dimensional paper-chromatography of the urine obtained after oral administration of the amine as summarized in Table I. These metabolites were conveniently numbered in order of R_f value on paper with *n*-butanol-acetic acid-water (4 : 1 : 5). Spots No. 1, 3 and 5 indicating negative reaction with Bratton-Marshall's reagents were not visible after acid or alkali hydrolysis of the urine. Furthermore, these three spots on the unsprayed paper were cut out under illumination by ultraviolet light, extracted with hot water, treated with *N* KOH for 15 minutes at 100°, neutralized with perchloric acid, and submitted to paper-chromatography after removal of potassium perchlorate. New spots thus obtained gave red color with Bratton-Marashall's reagent. The substances of spots No. 1 and 5 acquired the same R_f values of PABA while the spot No. 3 indicated that of PAHA. According to the coincidence of R_f values and the characterizations of the authentic acetyl derivatives of PABA and PAHA, the spots No. 1 and 3 were identified with AcPABA and AcPAHA respectively. The spot No. 5 was considered to be AcPABAG because it converted to PABA with hydrolysis and its eluate have purple color by naphthoresorcinol reaction

TABLE I

R_f Values and Color Reaction of PABA and its Metabolic Products

Spot No.	Name of substances	R_f		Color reaction	
		B-A-W[1]	M-B-B-W[2]	Bratton-Marshall's Reagent	Ehrlich's Reagent
1	*p*-*N*-Acetylaminobenzoic acid	0.90	0.83	—	Y (delayed)
2	*p*-Aminobenzoic acid	0.83	0.80	R-V	Y
3	*p*-*N*-Acetylaminohippuric acid	0.76	0.65	—	Y (delayed)
4	*p*-Aminohippuric acid	0.61	0.52	R-V	Y
5	*p*-*N*-Acetylaminobenzoyl glucuronide	0.55	0.62	—	Or-Y(delayed)
6	*p*-Aminobenzoyl glucuronide	0.43	0.47	R	Or-Y

1) Solvent system ; *n*-butanol-acetic acid-water (4 : 1 : 5)

2) Solvent system ; methanol-benzene-*n*-butanol-water (2 : 1 : 1 : 1)

In the table, the following abbreviation are used : red (R), violet (V), yellow (Y), orange (Or).

for glucuronic acid.

For the detection of glycine derivatives, a relatively large amount of the material was applied on the paper in a strip and a one-dimensional chromatogram was run in *n*-butanol-acetic acid-water (4 : 1 : 5). After the bands of developed amines were cut out and eluted with hot water, identification of amino acid was done chromatographically according to Nakao (*7*). Glycine was detected from the substances of spots No. 3 and 4. The R_f values and the color reactions of the spots No. 3 and 4 were agreed with those of synthetic AcPAHA and PAHA.

On the other hand, eluates from six spots were concentrated *in vacuo* and examined by naphthoresorcinol test. The presence of glucuronic acid was indicated in the two eluates from spots No. 5 and 6. Glucuronic conjugation were further confirmed by incubation with β-glucuronidase by Nakao's method (*11*). One glucuronide, the spot No. 6, gave a positive reaction with Bratton-Marshall's reagent and its character was identical with authentic PABAG. The spots No. 5 is probably of the type of *N*-acetylglucuronide. The results described above are summarized in Table I.

$S^{35}O_4$ was used for the detection of sulfate conjugate. About 100 μc. of isotope was given to a rabbit together with PABA by intravenous injection and the urine was desalted and analyzed by paper-chromatography. The paper treated with Bratton-Marshall's reagent was kept in contact with Fuji No-Screen Film in a cassette for 3 weeks. No spot was observed on the radioautogram as a metabolite of PABA.

Metabolic Fate of PABA in Several Mammals—PABA was given to human beings and dogs orally, to rabbits either orally or by intravenous injection and by intraperitoneal injection to mice. The urine excreted for 24 hours

TABLE II

Metabolic Products of PABA in Urine of Several Mammals

	Man	Dog	Rabbit		Mouse
	Oral	Oral	Oral	Intravenous	Intraperitoneal
PABA	±	++	±	+	++
AcPABA	++	−	+++	+	++
PAHA	+++	++	++	+++	++
AcPAHA	++	−	+	−	−
PABAG	+	+++	+++	+++	++
AcPABAG	++	−	++	−	−

thereafter were collected and desalted for two-dimensional paper-chromatography. The metabolic pattern of PABA among different kinds of mammals was shown qualitatively in Table II.

Fresh rabbit urine was collected from ureter after the administration of PABA by intravenous injection and submitted to the paper-chromatographic analysis. The greatest amount of free PABA was excreted immediately after its administration while it could hardly be detected in the ureter urine after more than 4 hours after its administration. Acetylated PABA could not be detected during one hour after the injection, since then its excretion occurred and continued for a long time. PAHA and PABAG overwhelmed other metabolites, and the maximum amount of those excreted substances was shown during 2 to 6 hours after intravenous injection. Any other metabolic product could not be detected in the ureter urine.

PABA Metabolism in Case of Simultaneous Administration of Glucose—When PABA and glucose was administered simultaneously to a rabbit and the collected urine was analyzed by paper-chromatography, seven spots due to PABA derivatives were seen on the paper, as shown in Fig. 1. Among them three large spots in this figure, A, B and C, after spraying with Bratton-Marshall's reagent were different from those of PABA metabolites detected in ordinary urine samples as described above. When spots A, B and C were cut out from unsprayed paper under the ultraviolet light and extracted with water, and the residue obtained from the extracts were submitted to a second chromatography, PABA, PAHA, and PABAG could be detected respectively. On the one-dimensional chromatogram run in metanol-benzene-butanol-water, three spots could be stained by spraying with Bratton-Marshall's reagent only after acid hydrolysis.

These substances could be detected by means of paper-chromatography in the rabbit urine excreted during a short time after injection, but they were hardly detectable in the urine stored for several hours in the bladder. Three spots overlapped between R_f values of 0.2 to 0.4 on the paper developed with neutral solvent system, and showed sugar reaction by spraying with benzidine reagent as well as with TTC reagent. Besides, all the eluates

from these spots with water gave blue color with commercial Tes-tape and this elucidated the presence of glucose. After drying the paper by hot air,

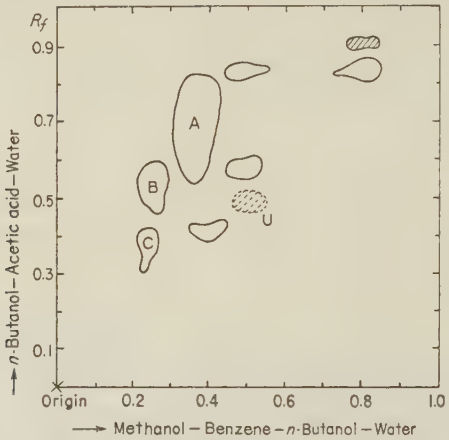

FIG. 1. Chromatogram of derivatives of PABA in rabbit urine after simultaneous injection of PABA and glucose. Solvents were methanol-benzene-*n*-butanol-water (2 : 1 : 1 : 1), followed by *n*-butanol-acetic acid-water (4 : 1 : 5). Two shaded spots were developed only with Ehrlich's reagent while the other spots with Bratton-Marshall's reagents. U represents the spots of urea.

these substances could be detected with glucose oxidase reagent on the one-dimensional chromatogram. From the agreement of the R_f values and of the color reaction with synthetic *N*-glucoside of PABA and PAHA, the spots A and B were identified with PABA-*N*-glucoside and PAHA-*N*-glucoside respectively. *N*-glucoside considered to be acid labile, and was decomposed when the chromatograms were run in acidic solvent, giving glucose and original amine. By various sugar reactions, glucose was detected in all the cases, below the three unknown spots with the R_f values of 0.23–0.30 on the two-dimensional chromatograms.

The following phenomenon must be especially taken care of; R_f values of PABA and its *N*-glucoside varied with the pH of original samples and in some cases, double spot was obtained on the paper. With the pH 6.0, R_f value of PABA was shown at 0.73 and 0.44, and two spots were connected with a trail. PABA-*N*-glucoside had two R_f values of 0.43 and 0.29 with the same pH. In case of PAHA-*N*-glucoside a wedge-shaped double spot with R_f values of 0.25 and 0.35 was obtained by salt effect. A large spot widened vertically on the two-dimensional chromatogram was obtained by a decomposition of *N*-glucosides occurred during the second development. After preparing these preliminary data, experimental results was discussed.

Analysis of Urine Collected from Ureter—Rabbit urine was collected directly from ureter through vinyl tube for the estimation of labile *N*-glucoside of

which decomposition possibly occurred in the bladder. The ureter urines were submitted to two-dimensional paper-chromatography and approximate determination of the metabolites was done by densitometry on the paper sprayed with sufficient amount of Bratton-Marshall's reagent, besides, colored spots were eluted with 0.2 *N* HCl, and the eluates were estimated by colorimetry. The relevant data in the ureter urines are given in Table III.

Table III

PABA Metabolites in Rabbit Urine Collected from Ureter after Simultaneous Administration of PABA and Glucose

No.	Time	Excreted amount in μg. per minute (calculated as PABA)									
		Total	PABA			PAHA			PABAG		
			Free	Glucoside	Total	Free	Glucoside	Total	Free	Glucoside	Total
1	0–15 min.	5,280	260	4,860	5,120	30	—	30	110	30	140
2	–35 ,,	5,280	370	4,060	4,440	110	370	480	210	110	320
3	– 1.0 hr.	5,750	580	4,200	4,770	170	690	860	400	230	630
4	– 1.5 ,,	1,980	240	830	1,070	100	480	570	200	100	300
5	– 2.0 ,,	1,250	130	500	630	40	440	480	60	50	110
6	– 3.0 ,,	550	70	210	280	30	190	210	30	40	70
7	– 3.5 ,,	320	60	60	120	30	110	140	30	50	70
8	– 4.0 ,,	480	60	110	170	50	170	230	30	40	70
9	– 5.5 ,,	250	50	50	100	30	100	130	20	10	30
10	–17.5 ,,	90	40	20	60	10	5	20	5	0	5
11	–23.0 ,,	40	5	20	30	5	—	5	—	—	—

PABA-N-glucoside Metobolism in Rabbit—One tenth or two tenth g. of synthetic PABA-*N*-glucoside dissolved in 15 ml. of physiological saline solution was administered intravenously to rabbits. Relatively small amount of the drug was used to avoid secondary effect of glucose originated from *N*-glucoside.

Unchanged *N*-glucoside and a trace of PABA were detected in rabbit urine by the foregoing analytical procedure. It was concluded that PABA-*N*-glucoside was excreted rapidly in urine without further transformation.

Non-enzymic Reaction of Glucose with PABA—One tenth to five tenth per cent glucose was usually detected in rabbit urine after the administration of PABA with glucose. It is well known that glucose or other sugars is combined with aryl amines under suitable environments, *e.g.*, in organic medium such as ethanol. In acid aqueous solutions, the formation of *N*-glucoside from glucose and PABA was occurred and accelerated with increasing acidity, and increasing glucose concentration. With the pH 3.0, eighty per cent of PABA was

converted to the *N*-glucoside for 60 minutes at 37° (original concentrations: PABA 0.1 per cent, glucose 10.0 per cent). Above pH 7.0, however, any glucoside could not be detected in reaction mixtures even in a high content of glucose. A similar tendency was ascertained in catheter urines instead of water and it was consequently considered that there was no possibility of conjugation of glucose and PABA in excreted urine.

DISCUSSION

By the method of counter current distribution analysis Tabor and his co-workers (*1*) and U m e d a (*2*) reported six metabolic products of PABA in human and rabbit urine. Identification of PABA metabolites and the difference of PABA metabolism in several mammals were studied paper-chromatographically in this work.

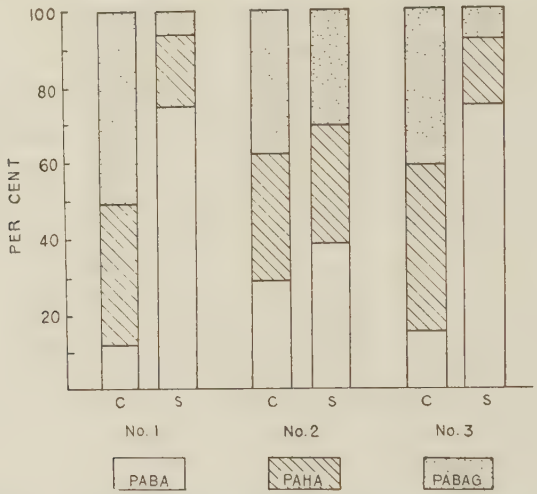

FIG. 2. Comparison between amines in rabbit urine after simultaneous injection of PABA and glucose and those after single injection of PABA (every dose of PABA was 1 g.). Each samples were 3 1/2 hours urine from bladder (No. 1, 2) and from ureter (No. 3).

According to this method excreted amount of AcPAHA and AcPABAG in human urine and that of AcPABAG in rabbit urine were more than that of the results by the above-mentioned authors', though similar results were qualitatively obtained. Acetylated PABA was generally detected in urine after one hour since the administration of the drug, while in the case of human urine, a large quantity of AcPABAG was obtained in a time when the excretion of PABAG was considerably little. On the other hand, however, *N*-sulfate or other labile metabolites could not be detected.

N-Glucoside of amine, a metabolic product of PABA, was detected in

rabbit urine after the simultaneous administration of PABA and glucose. *N*-conjugation of PABA with glucose was found non-enzymatically in acidic medium, and in this case an extremely higher concentration of glucose compared with PABA was needed. As a matter of course, such a condition was not necessary in urinary duct. Nevertheless, most of the metabolites were detected as *N*-glucoside just after excreted from the kidney. *N*-glucoside was rarely found in the urine excreted naturally, and this phenomenon seemed to be caused by its decomposition in the bladder.

According to the results of the determination of PABA, PAHA, and PABAG, these three substances in the urine after the administration of glucose, whether or not *N*-glucoside was detected, were entirely different from those without its administration; excreted amount of PABA was remarkably large compared with other metabolites. These results were shown schematically in Fig. 2. Although the ratio between excreted amount of PABA and other metabolites differed in each case, the same results were obtained in a rabbit either in the naturally collected urine or in the urine obtained from the ureter. Accordingly, these results could not be taken to be influenced by operation.

When glucose was contained in blood in high concentration, PABA was excreted in urine probably as *N*-glucoside, rather the taking another metabolic course, and then seemed to be decomposed again into PABA and glucose. This was endorsed by the experiment that the synthetic PABA-*N*-glucoside was excreted rapidly without any change. Meanwhile *N*-glucoside was not detected in urine without the administration of glucose, and the evidence of its enzymic formation was failed using rabbit tissue slice*. Booth and his collaborators (*12*) pointed out that *N*-glucoside was produced in Ringer solution as the artifact conjugated from naphthylamine and glucose. The mechanism of the formation of *N*-glucoside in animal body should be further studied in detail.

SUMMARY

After administration of *p*-aminobenzoic acid to human beings, dogs, rabbits and mouse, metabolic products contained in urines were identified paper-chromatographically. When *p*-aminobenzoic acid and glucose was administered simultaneously to a rabbit, *N*-glucoside combined to three amines (*p*-aminobenzoic acid, *p*-aminohippuric acid and *p*-aminobenzoyl glucuronide) were detected in the urine.

We wish to thank Prof. H. Yoshikawa for his kind advice and constant leading.

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ACID-SOLUBLE PHOSPHATE COMPOUNDS OF THE CILIATE PROTOZOON TETRAHYMENA GELEII W

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In recent years the synchronous culture of the ciliate protozoon *Tetrahymena* has been accomplished by many authors and it has provided a good tool in the biochemical investigation of the mechanism of cell division (*1-7*). The fundamental role of nucleotides in the mechanism of cell division attracted the attention of many investigators, and Plesner reported the paper chromatographic evidence for the presence of mononucleotides of adenine, guanine, uridine and cytidine in the cells of *Tetrahymena* (*2*, *3*).

In the preceding paper (*9*) changes in the chemical composition of acid-soluble sugar compounds were demonstrated by the present author during the synchronous culture of *T. geleii* W and a more precise identification was awaited.

The present investigation was designed to obtain a general outline of the phosphate compounds present in the acid extract of *T. geleii*, both in the stationary and exponential phase.

MATERIALS AND METHODS

The Experimental Organism and Cultivation—The ciliate protozoon *Tetrahymena geleii* W was obtained through the courtesy of Dr. Ishii of the Hosei University. The culture medium was the same as reported previously (*9*) except that inorganic P^{32} was added in a concentration of 1 μc. per ml. The original sample of P^{32} received from the Radiochemical Center, Amersham, England was heated at 100° for 30 minutes and polyphosphate were hydrolyzed. After dilution to the appropriate strength, it was added to the culture medium, the pH value of which was unaffected by this addition. The cells were cultivated in the P^{32}-containing media at 25° in a Fernbach-type flask and were checked often for their population avoiding contamination.

Preparation of the Acid-Soluble Fraction—After about 50 hours of cultivation the cells in the stationary phase were collected by a mild centrifugation for a minute, washed 3 times with 0.5 per cent NaCl, and were extracted with 20 ml. of ice-cold 10 per cent perchloric acid in a Potter-Elvehjem homogenizer. The precipitated proteines were centrifuged, re-extracted with 10 ml. of 5 per cent perchloric acid, and again separated from the extract by centrifugation. The combined extracts were neutralized by addition

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of 2 *N* KOH with phenol red as the internal indicator. The resulting precipitate was centrifuged off in the cold. A large aliquot of the supernatant was taken for ion-exchange chromatography, and a small aliquot was analyzed for ribose content and optical density at 260 mμ which was used to calculate the yield of nucleotide during chromatography. The protein precipitate was weighed and subjected to nitrogen determination by the micro-Kjeldahl method.

Ion-Exchange Chromatography—Dowex-1, X-10, 200-400 mesh, in the formate form was used and the elution was carried out with the gradient elution system of formic acid and ammonium formate described by Hurlbert, *et al.* (*10*). A column of 15 cm. × 1 cm^2. (15-ml. resin bed) was used with a 500-ml. mixing volume. Fractions of 5.0 ml. per tube were collected with an automatic fraction collector. The five elution ranges were obtained by changing the reservoir to contain 1 *N* formic acid, 4 *N* formic acid, 0.2 *M* ammonium formate in 4 *N* formic acid, 0.4 *M* ammonium formate in 4 *N* formic acid, and 0.8 *M* ammonium formate in 4 *N* formic acid, at Tubes No. 15, 45, 160, 200 and 260, respectively. Each fraction was subjected to the determination of its optical density at 260 mμ and 275 mμ, and a 0.2-ml. aliquot was taken for the determination of radioactivity.

Paper Chromatography—Ascending paper chromatography by a solvent system of butyric acid-conc. ammonia-EDTA (*11*) was used for the separation of substances isolated by column chromatography. For qualitative estimations of nucleotides and sugar esters, the following systems were used: isopropanol-ammonium sulphate-phosphate buffer pH 6.8 (2 : 60 *g.* : 100) (*12*), butyric acid-conc. ammonia-EDTA* (66 : 1 : 33) (*11*), and *tert*-butanol-picric acid-water (20 : 1 g. : 5) (*13*). For chemical analysis, the spots were cut out and eluted with water over night, then the eluates were passed through a Dowex-1 (chloride-form) column (0.5 × 3 cm.) and eluted with dilute HCl according to the method of Cohn and Carter (*14*). The eluate was examined for absorption in the ultraviolet region, analyzed for phosphate and pentose content, periodate consumption, acid lability of phosphate, and the specific activity of P^{32}.

Analytical Method—Optical density was measured in a 1.00-cm. quartz cuvette in a Hitachi Model DU Spectrophotometer. Absorption spectra of nucleotides were determined over the range of 220 mμ to 300 mμ, in both pH 1–2 and 11–12, and were compared with those of authentic samples. For the total phosphate determination, the samples were digested with 1 ml. of 60 per cent perchloric acid at 150° for 2 hours and phosphate was determined by the method of Horecker (*15*), after cooling. The acid lability was determined by the increase of inorganic P^{32} in *N* H_2SO_4 during the course of heating at 100° for 0–60 minutes. Inorganic phosphate was separated from organic phosphate by extraction with isobutanol in the presence of sodium molybdate and H_2SO_4 (*16*). The content of pentose was determined by the orcinol test according to Mejbaum as modified by Albaum and Umbreit (*17*) using a 45-minutes heating in a boiling water bath. Periodate oxidation was performed by the slightly modified method (*28*) of Dixon and Lipkin (*27*) for the estimation of 5′-substitution of pentose. The following color reactions of sugars were used for the determination of an unknown sugar-containing compound, total sugar by the method of Dische (*18*) and Dische-Popper (*19*), hexose by the primary cystein-H_2SO_4 reaction of Dische (*20*), mannose by the method of Dische-Shettles-Osnos (*21*), ketosugar by the method of Dische-Borenfreund (*22*) and hexuronic acid by the method of Dische (*23*). Cyanide complex formation for the

* The following abbreviation are used: AMP, adenosine monophosphate: GMP, guanosine monophosphate: CMP, cytidine monophosphate: UMP, uridine monophosphate: ADP, adenosine diphosphate: ATP, adenosine triphosphate: GTP, guanosine triphosphate: UTP, uridine triphosphate: DPN, diphosphopyridine nucleotide: RNA, ribonucleic acid: EDTA, ethylenediamine tetraacetic acid.

identification of DPN was carried out in 1 *M* NaCN according to the method of Colowick, Kaplan, and Ciotti (*24*), using the increase in the absorption at 325 mμ as a measure for *N*-substituted nicotinamide derivatives.

Determination of Specific Activity—For the determination of P^{32} content, a 0.2-ml. aliquot was taken, placed on a counting dish, dried under an infra-red lamp, and the radioactivity counted by a Riken Model 32 counter for a sufficient length of time to insure less than 5 per cent provable error.

RESULTS AND DISCUSSION

The acid-soluble phosphate compounds were extracted from the thoroughly washed cells of *T. geleii* W and the extract was submitted to column

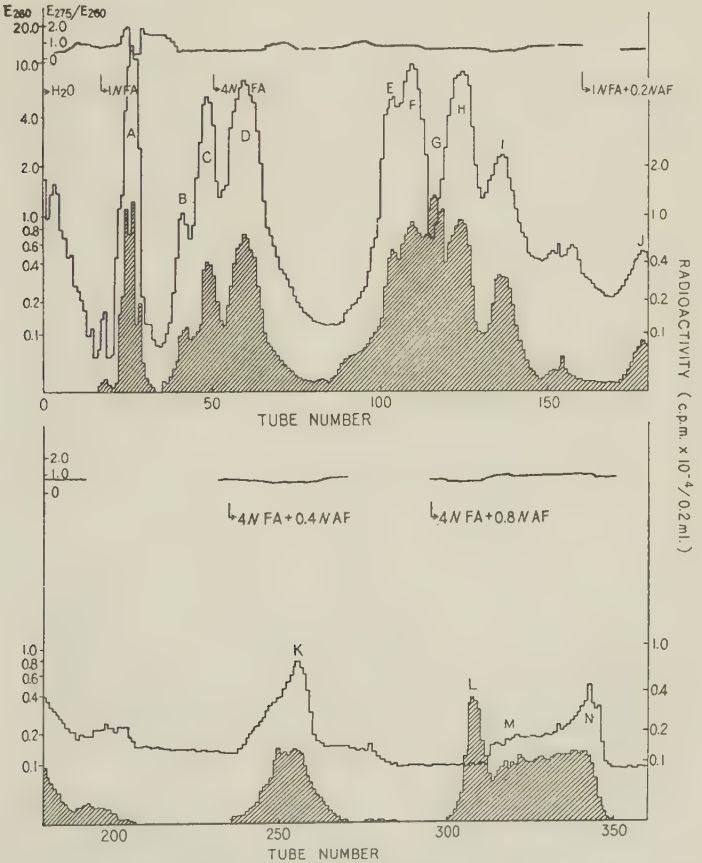

FIG. 1. Chromatography of the acid-soluble fraction of *Tetrahymena geleii* W by the formic acid system.

The optical density at 260 mμ is plotted for each fraction. The upper scale shows the ratio of the optical density at 275 mμ to that at 260 mμ. Shadowed area shows the radioactivity of each fraction.

chromatography of the formic acid system. The so obtained chromatogram is shown in Fig. 1. The optical density at 260 mμ and radioactivity of the effluent fractions were plotted against effluent volume. The eluted position and the absorbance ratio, E^{275}/E^{260}, gave a preliminary identification of each fraction. For further analysis, appropriate fractions were pooled and the solvent was removed by lyophilization or in some case the samples to be analyzed were precipitated directly from the eluates as barium salts. The samples thus obtained were decationized through Amberlite IR-120 (H-form), transformed into their acid-form, and subjected to paper chromatography for the establishment of purity of the peaks and comparison of their chromatographic behavior with those of the corresponding authentic nucleotides. Table I shows R_f values of the main components of each fraction.

TABLE I

R_f Values of Effluent Fractions

Fraction	(I)	(II)	(III)	Identification
A	0.67			CMP
B	0.63	0.35		5′-AMP
C	0.72	0.35		2′-AMP
D	0.72	0.23		3′-AMP
E	0.47	0.44	0.28	5′-GMP
F	0.51	0.31	0.34	3′-GMP
G	0.49	1.00	0.76	Orthophosphate
H	0.50	0.69		5′UMP
	0.50	0.54		3′UMP
I	0.60	0.30		ADP
K	0.52	0.37		ATP
L	0.32	0.00	0.00	?
	0.29	0.20	0.34	?
M	0.26	0.52		GTP
N	0.27	0.71		UTP

Solvent systems: (I) Butyric acid-conc. ammonia-EDTA (66 : 1 : 33). (II) Isopropanol-ammonium sulphate-phosphate buffer (pH 6.8) (2 : 60 g. : 100). (III) tert-butanol-picric acid-water (20 : 1 g. : 5). The filter paper was Toyo Roshi No. 53. All experiments were performed by ascending paper chromatography at about 20°.

Further purification of the phosphate compounds was carried out by paper chromatography, using *n*-butyric acid-conc. ammonia-EDTA (66 : 1 : 33) (*11*) (a) as the developing system. The developed bands were detected by ultraviolet absorption or radioautography, and each band was cut out from the paper to be eluted with a small amount of water. The eluted samples were purified again through Dowex-1 (chloride form) resin with use of

dilute HCl as an elution agent according to the method of Cohn and Carter (*14*). These procedures eliminated the interfering substances. Examinations were made with these eluates for ultraviolet absorption spectra, contents of the base, pentose, phosphorus, and labile phosphorus, periodate consumption, and sugar identification. Table II shows the result of these tests.

TABLE II

Analysis of the Fractions of Acid-Soluble Nucleotides of Tetrahymena geleii W

Fraction	Spectrum type	Ribose per mole of base	Total phosphate per mole of base	Periodate consumption*
A	cytidine	—	1.12	+
B	adenine	0.89	1.00	+
C	adenine	1.21	1.12	+
D	adenine	1.05	1.05	—
E	guanine	1.10	1.05	—
F	guanine	1.12	0.91	+
H	uracil	—	1.00	±
I	adenine	—	—	
K	adenine	1.02	3.00	+
M	guanine			
N	uracil			

The chemical data are expressed in terms of mole of base determined spectrophotometrically. The mole of bases were calculated with the following values as the milimolar extinction coefficients: cytidine, 13.7 at 278 mμ: uridine, 9.9 at 260 mμ: guanosine, 13.3 at 256 mμ: and adenosine, 14.1 at 258 mμ.

* + denotes the sample which consumes periodate. — denotes no consumption of periodate. ± denotes the unclear result.

Peak A was found to be CMP and a trace amount of DPN. The latter was confirmed by the spectral change which was observed after addition of cyanide. This peak was separated by column chromatography into two peaks which were shown both on the radioactivity plot and on the E^{275}/E^{260} plot indicating the presence of two cytidine-containing substances. However, the two were not separated by paper chromatograph which gave a single spot. The results of the chromatography and chemical data established further the occurrence in peaks B, C and D of 5′-AMP, 2′-AMP and 3′-AMP, respectively. Periodate consumption for the estimation 5′-substitution and stability toward 1 *N* H_2SO_4 in boiling water confirmed the presence of 2′- and 3′-adenine mononucleotides. Analysis of the main compounds of peaks E and F gave spectra similar to that of guanosine and revealed the presence of 1 mole of phosphorus per mole of base. By paper chromatography, peak E gave the same R_f value as authentic 5′-GMP and 2′-GMP and peak F, 3′-GMP. The results of periodate consumption established the occurrence of 5′-nucleotide

in peak E and of 2′- or 3′-nucleotide in peak F, indicating the presence of 2′- or 3′-GMP in the acid-soluble fraction of *T. geleii* W.

Peak G was found to be mainly inorganic phosphate, GMP, UMP, and a trace amount of unidentified phosphate compound which showed no ultraviolet absorption. In the preceding work, a large amount of sugar ester was demonstrated in the acid-soluble fraction of *T. geleii* W. Contrary to expectation peaks E, F and G contained no phosphate ester of sugars such as glucose phosphate and fructose phosphate.

The main component of peak H was identified as 5′-UMP, 3′- and possibly 2′-UMP by paper chromatography and periodate consumption. The data presented here reveal the presence of 2′- and 3′-AMP, 3′- and possibly 2′-GMP and UMP in the acid-soluble extract of *T. geleii* W. As for the natural occurrence of 2′- and 3′-mononucleotides, opinions vary. Bergkvist reported to have found these nucleotides in the acid soluble fraction of plant tissues (*25*, *26*), but denied their exsistence in the living tissues. It is known that the slice, when incubated for a long time at 37°, develops autolysis, resulting in a degradation of RNA. To test the possible occurrence of autolysis during preparation of acid extract the evenly P^{32}-labeled cells were collected as quickly as possible and killed instantly in dry ice-ether without washing cells. Besides, the exponential phase of the cells was chosen because in this phase the cells are undergoing active cell-growth and there will be minimal dead population, so that minimal autolysis due to dead cells would be expected. The distribution of nucleotides is shown in Table III as a percentage both

TABLE III

Nucleotiae Distribution of Tetrahymena geleii W, Both in Stationary and Exponential Phases, by Ion-Exchange Chromatography of Perchloric Acid Extract

Main component of fraction	Percentage of total nucleotide	
	Stationary phase (%)	Exponential phase (%)
CMP	16.5	11.7
5′-AMP	0.7	1.4
2′-AMP	6.9	4.5
3′-AMP	14.8	10.5
5′-GMP	7.9	4.4
2′ (3′)-GMP	17.2	27.5
UMP	23.6	15.3
ADP	2.0	
fraction J	2.9	13.4
ATP	1.6	8.7
CTP and UTP	3.2	3.2

in exponential and stationary phase, indicating the presence of 2′- and 3′-

nucleotides also in exponential phase. It must be therefore concluded that these 2′- and 3′-substituted mononucleotides occur naturally in *T. geleii* cells. It has been reported that *T. geleii* contains a high level of RNA and maintains a high RNA turnover rate. (*2–4*, *7*). Therefore it is natural to assume that 2′- and 3′-mononucleotides are the natural degradation products of RNA in the cells and form a part of mononucleotide pool as a source of nucleic acid.

Peak I was found to be ADP. Treatment of this fraction with acid led to the formation of 5′-AMP. Peak K contained ATP, from which ADP and 5′-AMP were obtained after mild acid hydrolysis. The chemical analysis was consistent with authentic ATP. Peaks M and N were identified as GTP and UTP, respectively, both by paper chromatography and base determination with ultraviolet spectral analysis.

The analysis of peak L proved the presence of a phosphate compound which gave positive color reactions of sugars. This compound was precipitable as a barium salt, was not adsorbed by charcoal and showed no ultraviolet absorption indicating the absence of any type of bases. The reaction with α-naphthol-H_2SO_4 and indole-H_2SO_4 for total sugar test gave remarkable color developement. The maximal wave-length of the latter color was 485 mμ indicating deviation of a 5 mμ from that of glucose. Analysis of this fraction by the cysteine-carbazole-H_2SO_4 reaction of Dische and Borenfreund and hexuronic acid test of Dische showed the presence of another type of a compound. Ninhydrin reaction for amino acid and diphenylamine reaction for fructose failed to produce any color. Treatment of the compound with 1 *N* H_2SO_4 at 100° resulted in the release of all organic phosphorus in 12 minutes indicating that the whole phosphorus group in the molecule was acid-labile. Further analysis was attempted for purification by paper chromatography with several solvent systems. R_f values are shown in Table I, indicating the presence of two spots. They presumably originate from degradation during the procedure. Further detailed investigation is in progress.

SUMMARY

The acid-soluble extract of *Tetrahymena geleii* W was analyzed by column chromatography. The identification was based on ultraviolet absorption spectra and chromatographic methods, together with chemical and isotopic techniques.

Evidence is given for the presence of 5′-monophosphates of adenosine, guanosine, uridine, and cytidine besides ADP, ATP, UTP, and DPN. In addition, the existence of unidentified phosphate compound which showed sugar reaction was presented.

The natural occurrence of 2′- and 3′-mononucleotides was presented and discussed.

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HEAT DECOMPOSITION PRODUCT OF FLAVIN ADENINE DINUCLEOTIDE IN AQUEOUS SOLUTION

I. FORMATION AND CRYSTALLIZATION OF "FOURTH FLAVIN COMPOUND"

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For the separating determination of flavin compounds in animal tissue using both paper chromatography and the lumiflavin fluorescence method (*1*,*2*), the warm water extraction method (*1*) was found to be the most suitable. In this method, the freshly excised tissue is cut into small pieces, heated in water at 80° for 5 minutes, homogenized in a glass homogenizer, and then heated again at 80° for 15 minutes.

During the examination of the temperature for the extraction of flavins from animal tissues without hydrolysing FAD or FMN, Yagi (*3*), one of the authors found that the homogenate of the liver heated above 90° gives a new flavin derivative which has been tentatively named "fourth flavin compound" (FFC) besides ordinary three flavin compounds in the liver, *i.e.*, FAD, FMN, and FR. By paper chromatography using several solvent (*4*), FFC was found to be different from not only FAD, FMN, and FR, but also lyxoflavin (*5*), flavinyl glucoside (*6*), and FAD-X (*7*),

Using an aqueous solution of crude FAD, it was found that FAD was converted to FFC quantitatively, thus FFC was considered to be produced from FAD. FFC was also found to be changed to FMN by heating it in acidic medium.

From these observations, the chemical structure of FFC and its enzymic relation were interested.

To these directions, a series of works were performed.

In this paper, the condition of FFC formation from purified FAD, and its crystallization are described.

MATERIALS AND METHODS

Flavins—FAD was prepared from *Eremothecium ashbyii* by the method of Yagi *et al.* (*8*). Its purity was above 92 per cent, and it contained no other flavins, metals or nucleic acids. FMN used for the analysis of flavins was a product of Hoffman la Roche Co.

pH of FAD Solution—pH of an aqueous solution of FAD was maintained by using a

Abbreviation: flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; free riboflavin, FR.

buffer solution. The buffer was a mixture of each 1×10^{-2} *M* (final concentration) of citrate and veronal, pH of which was adjusted by the addition of 0.1 *N* HCl or 0.1 *N* NaOH.

Formation of FFC—An aqueous solution of FAD (1×10^{-4} *M*) was filled up in a glass ampule and heated in water or oil bath. After heating, the separating determination of flavins was practiced. The total amount of flavins was firstly determined by the lumiflavin fluorescence method (*2*) using riboflavin as a standard, or by the measurement of its absorption at 450 mμ. Then, flavins of the sample were separated on a filter paper (Toyo Roshi No. 51, 2×30 cm.) by using the upper layer of *n*-butanol : acetic acid : water mixture (4 : 1 : 5, *v/v*) as the mobile phase. R_f values of FAD and FFC were 0.03 and 0.13, respectively. After drying in a dark room, a part of the paper strip containing each flavin was cut out from the paper, and was estimated by the lumiflavin fluorescence method (*2*). Supposing that one mole of FFC gives one mole of lumiflavin, g. mole of each flavin was calculated.

RESULTS

I. Condition of the Formation of FFC from FAD

The conditions of the formation of FFC from FAD were studied with purified FAD.

Effect of pH—The formation of FFC from purified FAD by heating it at 100° for 1 hour was examined at various pH's. As shown in Table I, the optimum pH for the formation of FFC was found at pH 4.0-8.0, where other flavins such as FMN and FR were not detected in the solution except remained FAD. Above pH 9.0, increase of pH brought about a marked decrease of total flavin. Below pH 3.0, FMN in the solution was increased with decreasing pH, then the amount of FFC was decreased.

Table I

Effect of pH on the Formation of FFC from FAD

pH	2	3	4	5	6	7	8	9	10	11	12
FAD	73.2	72.3	72.3	73.2	71.5	71.5	70.5	67.4	57.6	14.9	0
FMN	10.8	2.4	0	0	0	0	0	0	0	0	0
FFC	16.0	25.3	27.7	26.8	28.5	28.5	29.5	28.1	25.9	6.7	0
Total flavin	100.0	100.0	100.0	100.0	100.0	100.0	100.0	95.5	83.5	21.6	0

FAD was dissolved in the buffer of indicated pH to be 1×10^{-4} *M*. After the heating at 100° for 1 hour, flavins were analyzed. Numbers indicate the per cent of mole/liter of flavin to the initial concentration of FAD.

Effect of Temperature—The formation of FFC from purified FAD was examined by heating FAD for 1 hour at various temperatures. As shown in Table II, below 60°, FFC was not formed. At 70°, a small amount of FFC was found in the solution. Above 80°, formation of FFC was increased with ascending temperature up to 130°. The fact that FAD was not decomposed to FFC at 80° in the homogenate of animal tissue may be explained by the influence of coexisted substances.

TABLE II

Effect of Temperature on the Formation of FFC from FAD

Temperature (°C)	60	70	80	90	100	110	120	130
FAD	100.0	92.5	75.0	45.3	40.0	36.2	31.5	2.0
FFC	0	7.5	25.0	54.7	60.0	63.8	68.5	98.0

FAD was dissolved in distilled water to be 1×10^{-4} *M*. After the heating at indicated temperature for 1 hour, flavins were analyzed. Numbers indicate the per cent of mole/liter of flavin to the initial concentration of FAD. Total flavin concentration is not decreased by the heating.

Effect of concentration of FAD—The rate of formation of FFC from FAD was tested using the aqueous solution of graduated concentration of FAD. Results were shown in Table III, which shows that the formation of FFC was decreased with increasing concentration of FAD. The difference of the rate of FFC formation between in Table I and Table III may be attributed to the inhibitory action of citrate-veronal buffer.

TABLE III

Effect of Concentration of FAD on the Formation of FFC from FAD

Concentration of FAD (*M*)	6.4×10^{-3}*	2.1×10^{-3}	7.1×10^{-4}	2.4×10^{-4}	0.8×10^{-4}
FAD	67.5	61.0	50.0	41.2	38.8
FFC	32.5	39.0	50.0	58.8	61.2

FAD was dissolved in distilled water to be the indicated concentration. After the heating at 100° for 1 hour, flavins were analyzed. Numbers indicate the per cent of mole/liter of flavin to the initial concentration of FAD. Total flavin concentration was not changed by heating.

* 5.0 mg./ml.

II. Preparation of FFC Crystal from FAD

Taking the above experimental results into consideration, the preparation of FFC from FAD was practiced as follows:

Formation and Partial Purification of FFC—Five hundred mg. of purified FAD were dissolved in distilled water (1l.) and pH of the solution was adjusted to 8.0 by addition of 0.01 *N* NaOH. This solution was transfered into the pressure bottles, and stoppered tightly. The bottle was heated in autoclave (steam pressure: 20 pounds) for 2 hours. After cooling, the solution was passed through a column (6 cm. in diameter) filled with 200 g. of Florisil (60-100 mesh) to a height of about 27 cm. FFC and FAD in the solution were adsorbed at the top of the column. After the adsorption, the column was washed consecutively with 4 liters of 2 per cent acetic acid and 4 liters of distilled water, then about 1 liter of 0.5 per cent pyridine aqueous solution, as described by Dimant *et al.* (*7*). Thus, the formed adenine derivatives were completely eluted with 2 per cent acetic acid and water. When washed with 0.5 per cent pyridine,

the adsorbed band of flavins was moved to the bottom of the column. Then, flavins were eluted with 5 per cent pyridine aqueous solution. After adjusting pH of the solution to 8.0, the solution of FFC was washed five times with its equal volume of chloroform to remove pyridine, and filtered through a filter paper previously moistened with water. The filtrate was concentrated and dried under a reduced pressure. By this step, 360 mg. of dried yellow powder was obtained, in which about 200 mg. of FFC were contained.

Further Purification—A powdered cellulose chromatography similar to the

FIG. 1. Crystals of FFC, magnified 900 times.

method of Whitby (*9*) was used. The dried FFC powder was dissolved again in 2 ml. of water, adsorbed on about 5 g. of powdered cellulose and then dried in a reduced pressure. A glass chromatographic tube (6×60 cm.) was filled with tamped powdered cellulose to a height of about 40 cm. The adsorbate of FFC was placed on the top of this column, and the mixture of *n*-propanol : *n*-butanol : water (2 : 2 : 1, *v/v*) was allowed to flow down at the rate of 18 ml./hour. After two days, FFC fraction was cut out, eluted with warm water, and filtered through a sintered glass filter (No. 4). The solution was concentrated to about 10 ml. under a reduced pressure and then lyophilized to dryness. About 180 mg. of FFC were obtained.

Crystallization of FFC—FFC powder thus obtained was dissolved in 80 per cent ethyl alcohol aqueous solution and stored in an ice box. The fine plate crystals as shown in Fig. 1 were collected.

SUMMARY

"Fourth flavin compound", which had been found in heated liver homogenate, was formed from purified FAD by heating its aqueous solution. The

conditions for optimum formation of FFC from FAD were found as follows:

1. Optimum pH existed at between pH 4 and 8.
2. FFC formation was increased with ascending temperature until 130°.
3. The formation of FFC decreased with increasing concentration of FAD.

Under the optimum conditions, FFC was produced from FAD, purified and crystallized.

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STUDIES ON PROTAMINES

VII. N-TERMINAL SEQUENCES OF CLUPEINE AND SALMINE

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Applying the DNP**-method to some protamines, it was revealed that the N-terminal residue of salmine from *Oncorhynchus keta* was exclusively occupied by proline, whereas clupeine specimens from *Clupea pallasii* were found to have both alanine and proline as the N-terminus to various extents (*1*, *2*).

In this paper, the results of the studies on the N-terminal sequences of the protamines, obtained after separating several DNP-peptides from the tryptic digests of DNP-protamines, are fully described.

EXPERIMENTAL

Materials

DNP-Derivatives—Hydrochlorides of DNP-salmine (dinitrophenylated by Method 1, having R*** value of 1.02), DNP-clupeine I (Method 1, 0.67) and DNP-clupeine II (Method 2, 0.79 and 0.83) were prepared by the procedures fully described in the previous paper (*2*). Several DNP-amino acids and DNP-peptides,**** used as control, were prepared in the same way (*2*).

Trypsin—Any one of the following three specimens of crystalline trypsin***** was used:

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** *2,4*-Dinitrophenyl. Amino acids and peptides are abbreviated according to Brand and Edsall (*3*) and Sanger (*4*).

*** The ratio of optical densities at 390/360 mμ of the absorption spectrum of the DNP-derivative measured in N HCl or in 1 per cent $NaHCO_3$. The value is characteristic of the DNP-N-terminal residue. Confer (*2*).

**** DNP-Glycylglycine was kindly supplied by Dr. C. Hashimoto; glycyl-L-phenylalanine by Dr. T. Tobita, College of General Education of this University. L-Arginyl-L-arginine and L-arginyl-L-arginyl-L-arginine were obtained by separating from the tryptic hydrolysate of clupeine through column chromatography (*5*).

***** The preparation of A.C. was donated by Dr. H. Uchida, Institute for Infectious Diseases of this University, and that of W.B.S.C. by Dr. T. Shimanouchi, Department of Chemistry, Faculty of Science of this University. When tested with benzoyl-L-phenylalanine hydroxamide, it was later found, that the trypsin preparations, except that of N.B.C., were contaminated with a slight chymotryptic activity. Nevertheless, such an amount of contamination was found not to affect the results described in this paper, based on results obtained by a chymotryptic digestion experiment.

Preparations from Armour and Company, Worthington Biochemical Sales Co. (2× Cryst., 50 per cent $MgSO_4$, TL. 395-6) and Nutritional Biochemical Corporation (2× Cryst., 50 per cent $MgSO_4$, 9551).

Analytical Method

Measurement of the Absorption Spectra of DNP-Derivatives—For the measurement of absorption bands in the near ultraviolet region, a Beckman spectrophotometer, model DU, was used. As the molar extinction coefficients for DNP-prolyl peptide and DNP-alanyl peptide or the mixture of both in *N* hydrochloric acid, the values reported in the previous paper (*2*) were used. They were found to be applicable respectively for those in ethanol-hydrochloric acid, in which the ethanol concentration was less than 40 per cent (*v/v*), and in 0.25 *N* acetic acid.

Determination of α-Amino Nitrogen—The amount of α-amino nitrogen in the tryptic digest was determined by Van Slyke's manometric method (*6*).

Determination of Arginine and Proline—Arginine and proline were determined colorimetrically by Sakaguchi method using oxine (*7*) and by Chinard's ninhydrin procedure (*8*), respectively.

Paper Chromatography of Amino Acids—One-dimensional ascending paper chromatography was carried out on the Toyo Roshi No. 50 filter paper. As a solvent, *n*-butanol-formic acid-water (75 : 15 : 10, *v/v*) was usually used, but *n*-butanol-acetic acid-water (4 : 1 : 1, *v/v*) was also employed in some cases. Amino acids developed on the paper were revealed by treatment with ninhydrin. Arginine was confirmed by the Sakaguchi test on paper (*9*) and proline by the isatin reaction (*10*).

Chromatographic Identification of DNP-Derivatives—DNP-Derivatives were identified mainly by running in *tert*-amyl alcohol saturated with 0.1 *M* phthalate buffer (pH 5.4) on the similarly buffered Toyo Roshi No. 51 filter paper (*11*).

Paper Electrophoresis—One-dimensional paper electrophoresis was performed under the same conditions as described previously (*2*). Continuous paper electrophoresis was carried out using an apparatus according to Durrum (*12*). The diagram of a paper curtain is shown in Fig. 1. It had 24 serrate tabs for "drip points." As carrier the Toyo Roshi No. 2 filter paper was used and the background electrolyte was 0.25 *N* acetic acid.

PROCEDURES AND RESULTS

Tryptic Digestion of DNP-Protamines and the Extraction of DNP-Peptides with Methanol—As a typical example DNP-salmine was treated as follows. Two hundreds mg. of DNP-salmine hydrochloride (calculated to be 32.3 μmoles from its amount of absorption of DNP-group) in 20 ml. of *M*/30 phosphate buffer (pH 7.6) were incubated at 30°. Five mg. of crystalline trypsin (preparation of Armour and Company, including $MgSO_4$) were added to the solution and the mixture was digested at 30°. At time intervals (0.17, 6, 24 and 40 hours) each aliquot (5.0 ml.) was removed from the reaction mixture. A half ml. of each removed solution was used for the α-amino nitrogen analysis by Van Slyke's method (*6*). The amounts of α-amino nitrogen found with tryptic digests are shown in Table I. To each remaining solution (4.5 ml.) one drop of 6 *N* sulfuric acid was added to acidify the solution until pH approximately 4.8 and thus stop the digestion, the mixture was then dried *in vacuo* in a desiccator over calcium chloride. The dried residue (corresponding to 45 mg. of original DNP-salmine hydrochloride) was extracted

several times with absolute methanol (25 ml. in total). The methanol extract was dried under reduced pressure and the remaining matter was dissolved in water. Of an aliquot of the solution the amount of absorption in the near ultraviolet region was measured to estimate the amount of DNP-peptides in methanol ("methanol-soluble DNP-peptide fraction") extracted from each tryptic digest. Summarized results of DNP-salmine and DNP-clupeine are also shown in Table I.

TABLE I

Amounts of Amino Nitrogen Released and the Changes in the Amounts of "Methanol-Soluble DNP-Peptide Fractions" during Tryptic Digestion

Time of tryptic hydrolysis (*hr.*)		0.17	6	24	40
DNP-Clupeine II	Amino-N found per 10 mg. of DNP-clupeine hydrochloride ($\mu g.$)[1]	300	304	316	–
	Amount of DNP-peptides extracted into methanol (%)[2]	80[3,a]	81[b]	98[4]	90(77[5,c])
DNP-Salmine	Amino-N found per 10 mg. of DNP-salmine hydrochloride ($\mu g.$)[1]	288	286	328	–
	Amount of DNP-peptides extracted into methanol (%)[2]	62	86	85	92

1) Corrections (*ca.* 4 μg. per 10 mg. sample) for the contribution from guanidyl nitrogen (*13*) were not made.

2) Usually a dried tryptic digest from 45 mg. of DNP-protamine hydrochloride was extracted with 25 ml. of absolute methanol.

3) In this case, the tryptic digest from 15 mg. of DNP-clupeine hydrochloride was treated with 25 ml. in total of absolute methanol.

4) In this case, the tryptic digest from 20 mg. of DNP-clupeine hydrochloride was treated with 25 ml. of absolute methanol.

5) A result with DNP-clupeine I.

a) The R values of the "methanol-soluble" and "methanol-insoluble" DNP-peptide fractions were found to be 0.83 and 0.78, respectively.

b) Similarly, 0.81 and 0.76.

c) Similarly, 0.66 and 0.56.

Fractionation of the "Methanol-Soluble DNP-Peptide Fraction" by Continuous Paper Electrophoresis—Examination of the "methanol-soluble DNP-peptide fraction" by one-dimensional paper electrophoresis in 0.25 N acetic acid always revealed the presence of two yellow spots of DNP-peptides moving towards the cathode. By ninhydrin test it was also found that all the "methanol-soluble fractions" were contaminated with considerable amounts of free arginine-containing peptides moving faster towards the cathode than the DNP-peptides. Each "methanol-soluble fraction" (2–5.5 μmoles) in $N/8$ acetic acid (1–2 ml.) was applied to the continuous paper electrophoresis and separated quantitatively into two components as DNP-peptide. It took 20–30 hours to complete the separation. A typical paper electrophoretic pattern of

"methanol-soluble DNP-peptides" is shown in Fig. 1. The DNP-peptides were thus separated into two components, one consisting of that collected in tubes Nos. 10–12 (designated as DNP-band A) and the other in those Nos. 15–18 (DNP-band B). The fraction collected in tubes Nos. 16–24 gave a

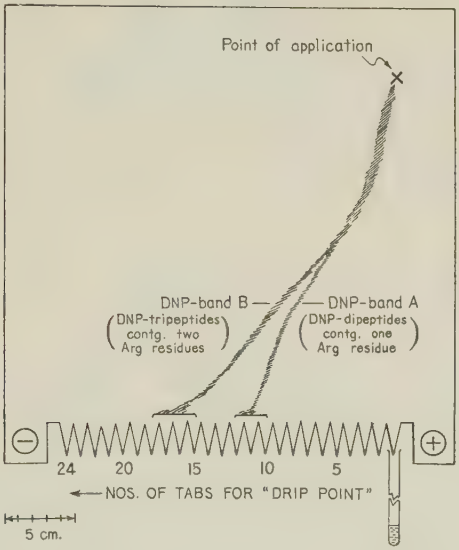

FIG. 1. Fractionation of the "methanol-soluble DNP-peptide fraction, obtained from the 40-hour tryptic digest of DNP-clupeine II, by continuous paper electrophoresis. Conditions: 0.25 *N* acetic acid as background electrolyte; 450 v., 2–2.5 mA.; the Toyo Roshi No. 2 filter paper as paper curtain.

positive ninhydrin test, showing that considerable amounts of free arginine-containing peptides were also contained. The amount of DNP-peptide in each tube (2.5–4 ml.) was estimated from its absorption in the near ultraviolet region. Then the contents of the tubes corresponding to the component A or B were combined respectively and dried under reduced pressure. Fig. 2 shows the distribution in percentage of the DNP-peptides in each tube after continuous paper electrophoresis. It also indicates the changes in the amount of each component as the tryptic digestion proceeded. As calculated from Fig. 2, the total recoveries of DNP-groups in this electrophoretic procedure amounted to 90–95 per cent.

Further Purification and Fractionation of Each of the DNP-Bands A and B by Talc Column Chromatography—Each DNP-band obtained by the above procedure was contaminated with some amounts of free arginine-containing peptides. The contamination was serious with the DNP-band B. The contaminant free peptides were removed through talc column chromatography. It was found by chance with DNP-peptides obtained from DNP-clupeine, that the DNP-prolyl peptides were less firmly adsorbed on talc than the DNP-alanyl

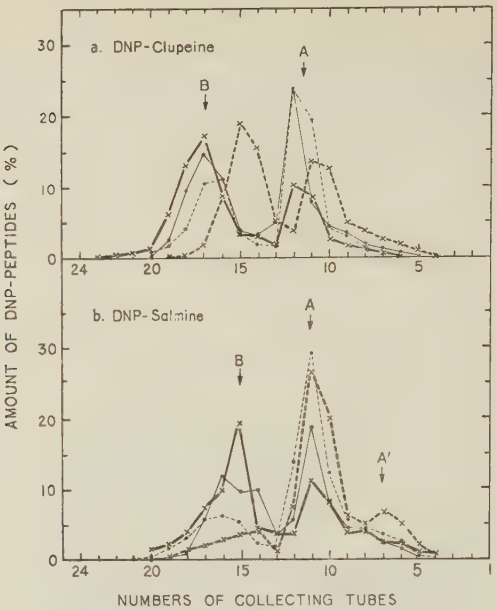

FIG. 2. Amount of DNP-peptides (as percentage of that applied to the paper) recovered from the methanol extracts of the tryptic digests of DNP-clupeine I (a; 40-hour digest), DNP-clupeine II (a; 0.17-, 6- and 24-hour digests) and DNP-salmine (b) following continuous paper electrophoresis.

As identified in the text, A and B denote DNP-peptides containing one arginine residue and two, respectively. In both cases a and b, —×— shows the pattern obtained from the digest of 0.17 hour, —·— 6 hours, ---·--- 24 hours and - - -×- - - 40 hours, respectively.

ones, thus both types of DNP-peptides being easily separated by this procedure. A typical example with DNP-peptides obtained by 40-hour tryptic digestion of DNP-clupeine (with R value of 0.79) was follows.

DNP-Peptides (4.40 μmoles, $R=0.78$) obtained by methanol extraction as described above (the extraction yield of the DNP-groups, 90 per cent) were separated by continuous paper electrophoresis into two components, DNP-bands A and B. The main fraction of DNP-band A (combined effluents in tubes Nos. 11–14, 2.20 μmoles from its DNP-group absorption), was dissolved in 1 ml. of water and was applied to the talc* column (ϕ 15×31 mm.). Subsequent washing and elution were carried out at 0°. The rate of flow of the eluent was adjusted by applying pressure to be approximately 1.7$_4$ ml. per 20 minutes and each 1.7$_4$ ml. fraction of the effluent was taken in a collecting tube. Free peptides containing arginine residue were rapidly washed down by N hydrochloric acid, while the DNP-peptides remained on

* A preparation of Kozakai Seiyaku Co., Ltd. Before use the talc was washed repeatedly by decantation with N HCl at 60°.

the top of the column, being strongly adsorbed on talc. Free peptides with positive Sakaguchi test were collected into tubes Nos. 4–9. After the effluent gave negative Sakaguchi reaction, the column with further 10 ml. of *N* hydrochloric acid. Then the column was washed with cold 1 : 4 (*v/v*) ethanol-*N* hydrochloric acid. A part (DNP-peptide A-I) of the DNP-peptides* was rapidly eluted by the medium. Thereafter, by 4 : 1 (*v/v*) ethanol-*N* hydrochloric acid, the remainder of the DNP-peptides (DNP-peptide A-II) was completely eluted. DNP-Peptides rapidly eluted by 1 : 4 and 4 : 1 (*v/v*) ethanol-*N* hydrochloric acid are generally designated as I and II, respectively. Each effluent was diluted with water and the absorption spectrum in the near ultraviolet region was measured to determine the amount of DNP-peptide

Table II

Talc Column Chromatography of DNP-Band A Obtained from DNP-Clupeine II.

Nos. of collecting tubes	Eluent	R[1] value of the effluent DNP-peptide	Amount of the DNP-peptide	Designation of the DNP-peptide	Amount of each DNP-peptide calculated from the R value	
					DNP-prolyl peptide	DNP-alanyl peptide
			(*μ mole*)		(*μ mole*)	(*μ mole*)
1–14	*N* HCl	—	0	—	—	—
15–19	1 : 4 (*v/v*) EtOH-*N* HCl	—	0	—	—	—
20–21	,,	1.00	0.90	A-I	0.90	0
22–24	,,	1.01	0.18	A-I	0.18	0
25–28	4 : 1 (*v/v*) EtOH-*N* HCl	0.62	0.89	A-II	0.13	0.76
29–32	,,	—	0.01	—	—	—
Total			1.98		1.21	0.76

DNP-Band A fraction, 2.20 *μ* moles, obtained by continuous paper electrophoresis of the methanol extract of the 40-hour tryptic digest of DNP-clupeine II (R value=0.79), was applied to the talc column (ϕ 15×31 mm.), washed and eluted by the media shown in the table. Each 1.7$_4$ ml. of the effluent was collected in a tube. For details see the text.

1) See the footnote of p. 82 (***).

* Later it was found that, on prolonged washing with *N* HCl, the DNP-band A from DNP-clupeine also was separated into two layers on talc column as was the case with the DNP-band B. In the former case, the lower, less firmly adsorbed layer corresponded to the DNP-peptide A-I which was found to be a DNP-prolyl peptide, and could easily be eluted with 1 : 4 (*v/v*) ethanol-*N* HCl. The remaining upper band, A-II, a DNP-alanyl peptide, was then rapidly eluted with 4 : 1 (*v/v*) ethanol-*N* HCl.

contained and to infer the DNP-N-terminal amino acid by calculating its R value from the curve. Each fraction was then dried under reduced pressure at 40°. Summarized results are listed in Table II. Fig. 3 shows the absorption spectra of the DNP-peptides A-I and A-II thus obtained.

As is clear from the R value of the DNP-peptide in the effluent fraction (see Table II) and from the shape of the absorption spectrum of each DNP-

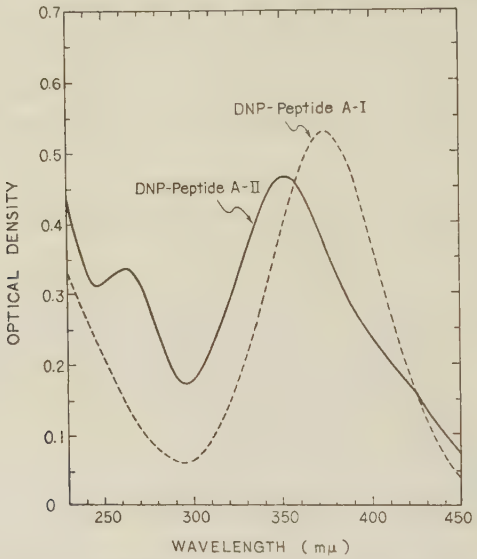

FIG. 3. Absorption spectra of DNP-peptides purified and fractionated by talc column chromatography.

Typical figures of DNP-peptides A-I and A-II, isolated from DNP-band A as summarized in Table II, are shown here. DNP-Peptide A-I from the combined effluent of tubes Nos. 20–21 was diluted with water to 30 ml. and the absorption was measured using a 1 cm. silica cuvette. DNP-Peptitde A-II fraction from tubes Nos. 25–28 was diluted to 30 ml. and similarly treated.

peptide (see Fig. 3), DNP-peptide A-I is identified as a DNP-prolyl peptide*. Similarly, in the case of clupeine, DNP-peptide A-II is considered to consist mainly of a DNP-alanyl peptide* with a contaminant amount of DNP-prolyl peptide (see also Table IV). From the R value of the DNP-peptide in each effluent fraction, the amounts of the DNP-prolyl and DNP-alanyl peptides were calculated**, as shown in the last two columns of Table II.

* As already described, an α-DNP-prolyl peptide and an α-DNP-alanyl peptide should have R values of 1.02 and 0.55, respectively (*2*).

** In these calculations, DNP-prolyl peptides with R values of 1.00 or more were considered to be 100 per cent with respect to DNP-N-terminal proline. Thus the calculations were made with DNP-peptides having R values of 0.56–0.99, according to the Fig. 5 in the previous paper (*2*).

The main fraction of DNP-band B (tubes Nos. 17–20, 1.76 μmoles of DNP-groups) was applied to the talc column (ϕ 15×34 mm.) and similarly treated. In this case, about 100 ml. of *N* hydrochloric acid were required to wash down the free peptides having positive Sakaguchi reaction. Through the washing, the DNP-peptides on the column separated clearly into two layers, the upper (corresponding to DNP-peptide B-II), reckoned from the top of the column, was 9 mm. thick, and the lower (DNP-peptide B-I) was between 11–13 mm. thick. The lower DNP-layer was easily eluted by 1:4 (*v/v*) ethanol-*N* hydrochloric acid. The remaining upper layer was then rapidly eluted by 4:1 (*v/v*) ethanol-*N* hydrochloric acid. These results are summarized in Table III. Also in this case, as can be seen from the *R* value of each fraction, DNP-peptide B-I consists of a DNP-prolyl peptide and B-II mainly of a DNP-alanyl one.

Table III

Talc Column Chromatography of DNP-Band B Obtained from DNP-Clupeine II.

Nos. of collecting tubes	Eluent	R[1] value of the effluent DNP-peptide	Amount of the DNP-peptide	Designation of the DNP-peptide	Amount of each DNP-peptide calculated from the R value	
					DNP-prolyl peptide	DNP-alanyl peptide
			(μ *mole*)		(μ *mole*)	(μ *mole*)
1–51	*N* HCl	—	0	—	—	—
52–54	1:4 (*v/v*) EtOH-*N* HCl	—	0	—	—	—
55–56	,,	1.01	0.18	B-I	0.18	0
57	,,	0.94	0.03	—	0.03	0.03
58	4:1 (*v/v*) EtOH-*N* HCl					
59	,,	0.69	0.03	—		
60	,,	0.59	1.12	B-II	0.09	1.03
61–63	,,	0.54	0.09		0	0.09
Total			1.45		0.30	1.15

DNP-Band B fraction, 1.76 μmoles, obtained by continuous paper electrophoresis of the methanol extract of the 40-hour tryptic digest of DNP-clupeine II (R value=0.79), was applied to the talc column (ϕ 15×34 mm.), washed and eluted by the media shown in the table. Each 1.7$_4$ ml. of the effluent was collected in a tube. For details see the text.

1) See the footnote of p. 82 (***).

The results similarly obtained with DNP-salmine and other specimens of DNP-clupeine under several conditions are also summarized in Table IV. In

Table IV

Talc Column Chromatographic Fractionation of DNP-Bands A and B Obtained from the Tryptic Digests of DNP-Protamines and the Determination of the Structure of the DNP-Peptides thus Fractionated.

DNP-Protamine	Time of tryptic digestion	Talc column chromatography of DNP-band: DNP-Band (Nos., μ mole, %)[2] applied to the talc column	Talc column chromatography of DNP-band: Designation, amount, $R^{1)}$ value and assigned N-terminal group§ of DNP-peptide recovered in each fraction	Amino acid composition of DNP-peptide after acid hydrolysis: Paper chromatographic identification	Amino acid composition of DNP-peptide after acid hydrolysis: Arg/DNP-group	Amino acid composition of DNP-peptide after acid hydrolysis: Pro/DNP-group	Structure of the DNP-peptide††
	(*hr.*)		(μ *mole*)		(*mole/mole*)	(*mole/mole*)	
DNP-Clupeine I ($R^{1)}$=0.67)	40	A (7-12, 0.68, 41)	A-I 0.17 0.98 DNP-Pro	Arg, Pro †	...	0.60	DNP-Pro·Arg
			A-II 0.48 0.63 DNP-Ala	Arg †	0.80	0.06	DNP-Ala·Arg
		B (13-17, 0.84, 50)	(B-I) 0 — —	—	—	—	—
			B-II 0.74 0.60 DNP-Ala	Arg †	2.1	0.15	DNP-Ala·Arg·Arg
DNP-Clupeine II ($R^{1)}$=0.83)	0.17	A (10-12, 0.32, 21)	A-I 0.25 0.96 DNP-Pro	Arg, Pro	1.0	...	DNP-Pro·Arg
			(A-II) 0 — —	—	—	—	—
		B (16-19, 0.75, 50)	B-I 0.26 1.00 DNP-Pro	Arg, Pro †	2.0	...	DNP-Pro·Arg·Arg
			B-II 0.47 0.59 DNP-Ala	Arg †	1.6	...	DNP-Ala·Arg·Arg
DNP-Clupeine II ($R^{1)}$=0.79)	40	A (11-14, 2.20, 50)	A-I 1.08 1.00 DNP-Pro §	Arg, Pro †	1.1	0.84	DNP-Pro·Arg
			A-II 0.89 0.62 DNP-Ala	Arg	1.0	...	DNP-Ala·Arg
		B (17-20, 1.76, 40)	B-I 0.18 1.01 DNP-Pro §	Arg, Pro	(2.9)	...	DNP-Pro·Arg·Arg
			B-II 1.12 0.59 DNP-Ala §	Arg †	1.9	0.11	DNP-Ala·Arg·Arg
DNP-Salmine ($R^{1)}$=1.02)	0.17	A' (5- 9, 0.59, 14)	A'-I 0.35 0.99 DNP-Pro	Arg, Pro †	0.90	...	DNP-Pro·Arg
			(A'-II) 0[3] — —	—	—	—	—
		A (10-12, 0.87, 23)	A-I 0.49 0.97 DNP-Pro	Arg, Pro †	1.5	0.95	DNP-Pro·Arg
			(A-II) 0[3] — —	—	—	—	—
		B (13-20, 2.0 , 52)	B-I 1.63 0.99 DNP-Pro	Arg, Pro †	(1.2)	1.0	DNP-Pro·Arg·Arg
			(B-II) 0.03 0.69 ...	...	...	...	...
	40	A' (6- 8, 0.98, 18)	A'-I 0.58 0.99 DNP-Pro §	Arg, Pro	1.0	0.79	DNP-Pro·Arg
			(A'-II) 0[3] — —	—	—	—	—
		A (10-12, 2.92, 54)	A-I 2.72 0.99 DNP-Pro §	Arg, Pro †	0.91	1.0	DNP-Pro·Arg
			(A-II) 0[3] — —	—	—	—	—
		B (14-18, 0.75, 14)	B-I 0.58 1.00 DNP-Pro	Arg, Pro †	2.1	...	DNP-Pro·Arg·Arg
			B-II {0.13 0.63 / 0.04 0.51} ...	...	...	...	...

... denotes that no determination was made. 1) See the footnote of p. 82 (***) 2) The values in the parenthesis denote in that order, the Nos. of collecting tubes in the continuous paper electrophoresis, the amount of the DNP-peptides in the fraction, and the percentage of the DNP-peptides to the amount applied to the continuous paper electrophoresis. 3) A slight amount of substance having an absorption band in the near ultraviolet region was found. But the absorption spectrum was not of a DNP-peptide type. § Generally identified from the $R^{1)}$ values. Only in the cases marked with §, identification of the DNP-compound after acid hydrolysis was also performed. † A very faint ninhydrin-positive spot corresponding to serine was observed on the paper chromatogram. But quantitatively, its amount was less than 0.1 mole per mole of DNP-group and so it was disregarded. †† Assignment was made for the main component of the DNP-peptide, since DNP-peptide of type I from DNP-clupeine was yet usually ontaminated with DNP-peptide of type II (at max. 10-20 per cent), and *vice versa*.

the case of DNP-salmine, the DNP-band A sometimes showed a "subpeak" in its anode side (see Fig. 2), therefore, this fraction, designated as A′, was collected and treated separately.

Identification of DNP-Peptides Separated in the Above—As seen in Tables II, III and IV, four DNP-peptides—A-I, A-II, B-I and B-II—were isolated from the tryptic digests of DNP-clupeine, while two, A-I and B-I*, from those of

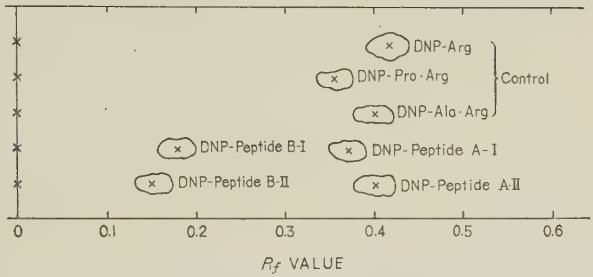

FIG. 4. One-dimensional paper chromatography of four DNP-peptides isolated from the tryptic digest of DNP-clupeine. Descending paper chromatography was carried out, using the solvent system of *tert*-amyl alcohol-0.1 *M* phthalate buffer (pH 5.4) (*11*), on the similarly buffered Toyo Roshi No. 51 paper. R_f values were found somewhat to fluctuate in each chromatogram. For details of the isolation of these DNP-peptides see the text.

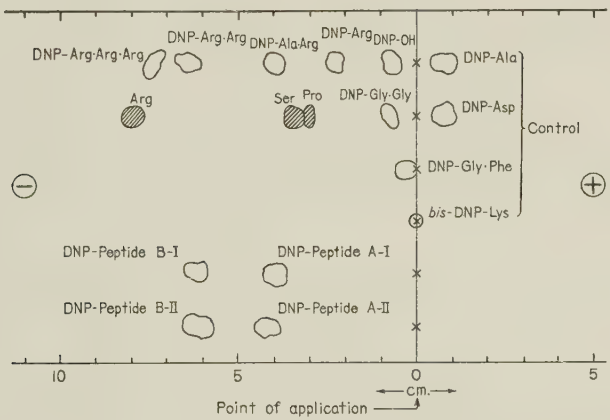

FIG. 5. Paper electrophoretic pattern of four DNP-peptides isolated from the tryptic digest of DNP-clupeine. Electrophoresis was performed for 45 minutes on the Toyo Roshi No. 51 filter paper in 0.25 *N* acetic acid applying 20 v/cm. and 0.25 mA./cm. Shaded portions in the figure denote that these spots were revealed by ninhydrin reaction.

DNP-salmine. Fig. 4 shows the pattern obtained on one-dimensional paper

* A fraction with characteristics of B-II was also isolated from the 40-hour digest (see Table IV), but it has not been pursued further as the amount was too small.

chromatography of the DNP-peptides from DNP-clupeine by running in *tert*-amyl alcohol-0.1 *M* phthalate buffer system (*11*), which was found to be a very satisfactory system for DNP-peptides containing arginine. Fig. 5 indicates the pattern of these DNP-peptides obtained by one-dimensional paper electrophoresis in 0.25 *N* acetic acid. In both figures, the patterns of several authentic DNP-compounds are shown together for control.

The structures of these DNP-peptides were determined by the following procedures. DNP-Peptides were hydrolyzed at 110° in sealed tubes with 6 *N* hydrochloric acid for 24 hours. The hydrolyzates were dried in a vacuum desiccator over sodium hydroxide. Each residue was dissolved in water. Of aliquots of the solution the amounts of arginine and proline in the hydrolyzate were determined by applying the Sakaguchi reaction (*7*) and Chinard's method (*8*), respectively. Thus the molar ratios of arginine and proline to the original DNP-group subjected to hydrolysis were estimated. Other aliquots from the solution were applied to one-dimensional paper chromatography to identify the constituent amino acids in the hydrolyzate. The presence of proline was examined by the isatin test on the paper chromatogram, and at the same time, its amount was also estimated semi-quantitatively from its intensity of blue color. The DNP-N-terminal amino acids of these DNP-peptides could easily be inferred from the shapes (see Fig. 3) and *R* values (see Tables II-IV) of their absorption spectra in the near ultraviolet region. When there remained enough DNP-peptides, the DNP-N-terminal amino acids were also identified paper chromatographically after acid hydrolysis (6 *N* HCl, 100°, 8 hours in sealed tubes). As already reported (*2*, *14*), DNP-proline and DNP-prolyl peptides are so labile to acid hydrolysis in 6 *N* hydrochloric acid as to yield quantitatively 2, 4-dinitrophenol and proline. Therefore, the presence of a stoichiometric amount of proline in the acid hydrolyzates of DNP-prolyl peptides may safely be considered to come from the DNP-N-terminal proline. The results obtained through above procedures with these DNP-peptides and their concluded structures are summarized in Table IV.

Examination of DNP-Peptides Not Extracted into Methanol—To study the longer N-terminal amino acid sequences, DNP-peptides which were not extracted into methanol were examined similarly. Each "methanol-insoluble fraction" applied to the talc column was freed from arginine-containing free peptides by prolonged washing with *N* hydrochloric acid, and the DNP-peptides remained on the talc were eluted separately with 1 : 4 and 4 : 1 (*v*/*v*) ethanol-*N* hydrochloric acid as described before. Attempts to determine the structures of the DNP-peptide fractions thus obtained were not successful, as all the component amino acids of the starting protamines were usually found in each of their acid hydrolyzates. It was also found that "methanol-insoluble fractions" had smaller *R* values than those of original DNP-protamine (see Tables I and IV). Some parts of the results will be quoted in the next section.

*Calculation of the Molar Ratio of N-Terminal Proline to Alanine of Clupeine**— From the *R* value (0.67) of DNP-clupeine I, the molar ratio of N-terminal proline to alanine of this clupeine specimen was estimated to be 0.25 : 0.75. Calculated from the values in Table IV, the molar fraction of DNP-prolyl peptide in DNP-clupeine I was estimated to be approximately 0.17**. Also, the molar fraction of N-terminal proline of clupeine II is approximately 0.60 from its *R* value of 0.83. Similar calculation using the results of 0.17-hour digestion of DNP-clupeine II, gave approximately 0.50 as the molar fraction of N-terminal proline of clupeine II***.

DISCUSSION

The rate of hydrolysis of DNP-protamines by trypsin is shown in Table I. This shows, that the splitting of the most part of the peptide-bonds was essentially complete within 6 hours ; however, some parts of the bonds require as long as 24 hours or more for complete hydrolysis. The yields of DNP-peptides soluble in absolute methanol increased from 60 to about 100 per cent as the hydrolysis proceeded.

The methanol-soluble DNP-peptides were separated by paper electrophoretic technique into two groups, the one (DNP-band A), containing one arginine residue, and the other (DNP-band B), containing two arginine

* In the following calculation, it was generally assumed, that the losses of DNP-peptides in the course of purification and isolation procedures occurred equally both with DNP-prolyl peptide and the DNP-alanyl one. As was described in the footnote (**) of p. 88, some correction with the real contents of DNP-prolyl and/or DNP-alanyl peptide was made taking into account the *R* value of each DNP-peptide.

** After 40-hour tryptic hydrolysis, 77 per cent of the original DNP-groups in DNP-clupeine I were extracted into the methanol-soluble fraction (see Table I). The fraction was further separated into three parts, *i.e.* A-I, A-II and B-II (see Table IV). DNP-Peptide A-I with *R* value of 0.98 was computed to contain approximately 0.91 molar fraction of DNP-prolyl peptide (confer Fig. 5 in the previous paper (*2*)); similarly, DNP-peptides A-II (*R* value=0.63) and B-II (0.60) contain 0.17 and 0.10 molar fraction of DNP-prolyl peptide, respectively. Thus the total amount of DNP-prolyl peptide and that of the DNP-alanyl one, finally isolated from the methanol-soluble fraction, were calculated as $(0.17\times0.91)+(0.48\times0.17)+(0.74\times0.10)=0.31\,\mu$ mole and $(0.17\times0.09)+(0.48\times0.83)+(0.74\times0.90)=1.08\,\mu$ mole, respectively. An *R* value of 0.56 (see foot note c) of Table I) was observed for the methanol-insoluble fraction, from which nearly no DNP-prolyl peptide was isolated on talc column chromatography. Therefore, the molar fraction of DNP-prolyl peptide was calculated to be : $0.77\times0.31/(0.31+1.08)=0.17$.

*** From the methanol-soluble fraction containing 80 per cent of the original DNP-group, $(0.25\times0.87)+0.26+(0.47\times0.08)=0.52\,\mu$ mole of DNP-prolyl peptide and $(0.25\times0.13)+(0.47\times0.92)=0.46\,\mu$ mole of DNP-alanyl one were finally obtained (see Tables I and IV). The methanol-insoluble fraction had an *R* value of 0.78, and on talc column chromatography, a molar fraction of approximately 0.38 was attributed to the DNP-prolyl peptide in that fraction. Consequently, the molar fraction of whole N-terminal proline of clupeine II was calculated to be : $0.80\times0.52/(0.52+0.46)+(0.20\times0.38)$ 0.50.

residues. By talc column chromatography, each group of the DNP-bands was, in general, further separated into DNP-prolyl and DNP-non-prolyl peptides. Thus, as shown in Tables II-IV, the following four DNP-peptides in total, DNP-Pro·Arg (A-I), DNP-Ala·Arg (A-II), DNP-Pro·Arg·Arg (B-I) and DNP-Ala·Arg·Arg (B-II), were isolated from the tryptic digests of DNP-clupeine II. Similarly only two DNP-peptides, DNP-Pro·Arg (A-I) and DNP-Pro·Arg·Arg (B-I) were obtained from the tryptic digests of DNP-salmine. In view of the reasonable recoveries of these DNP-peptides as well as of the mode of change in the amount of each DNP-peptide during the tryptic digestion (*cf.* Tables II-IV and Fig. 2), it may be concluded* that salmine should have an N-terminal sequence of Pro·Arg·Arg···, while clupeine II should have two sorts of N-terminal structures, Pro·Arg·Arg··· and Ala·Arg·Arg···. Clupeine I, with which only one case of 40-hour tryptic digestion was observed, may also have the same two kinds of N-terminal sequences as clupeine II.

Monier and Jutisz (*24*) demonstrated with a commercial specimen of salmine from *Oncorhynchus* salmon, that the protamine has an N-terminal structure of Pro·Arg·Arg. Our result with salmine from *Oncorhynchus keta* salmon is quite consistent with that of Monier and Jutisz. Felix (*15*, *17*) reported that protamines from several species of *Salmonidae*, such as salmine from *Salmo salar*, iridine from *Salmo irideus*, truttine from *Salmo trutta* and fontinine from *Salmo fontinalis*, have the same N-terminal structure of Pro·Val·Arg···. According to the view of Felix, this N-terminal structure is characteristic of these protamines of *Salmonidae*. The results of the present and French authors with salmine from *Oncorhynchus* salmon indicate, that such a sequence is not a general characteristic of all *Salmonidae*. According to Felix (*15*, *17*), clupeine from *Clupea harengus* has the N-terminal sequence of Pro·Ala·Arg···, while in contrast, no such a sequence was ever found in our specimens of clupeine from *Clupea pallasii*.

In these experiments, no DNP-peptides containing three or more arginine residues could be found in the methanol-soluble fraction**. Furthermore, all the attempts to obtain such longer N-terminal peptides from each methanol-insoluble fraction failed to give definite results, as every fraction of DNP-peptides usually gave all kinds of component amino acids of each original protamine.

* The inhomogeneous nature of several kinds of protamines (*e.g.* clupeine, salmine, iridine and mugiline β) has recently been demonstrated by various physical and/or chemical means (*15-23*). The methods used in this N-terminal sequence study do not necessarily support the assumption, that all DNP-dipeptides, DNP-Pro·Arg and DNP-Ala·Arg, were derived from the corresponding DNP-tripeptides, DNP-Pro·Arg·Arg and DNP-Ala·Arg·Arg, respectively. Therefore, strictly speaking, the possibility, that some fractions of the third amino acid in the N-terminal sequence of salmine and clupeine may be located by amino acids other than arginine, is not entirely excluded. Nevertheless, no positive evidence for the possibility was obtained in our study using an enzymic technique.

** Ando *et al.* (*25*) previously reported that, from the results obtained following leucine aminopeptidase digestion, it is probable that a serine residue occupies the fourth position from the N-terminus of the alanyl chain of our clupeine specimen.

Examination of the results obtained following the tryptic hydrolysis of DNP-protamines, shows, that DNP-Pro·Arg·Arg is more easily degraded into the DNP-dipeptide (DNP-Pro·Arg) than DNP-Ala·Arg·Arg into DNP-Ala·Arg. This is a fact of interest and may be explained as the result of different effects of proline and alanine residues on the susceptibility of their neighbouring peptide bonds to tryptic hydrolysis (see Table IV and Figs. 2-a and 2-b).

In the previous paper (*2*) it was reported that our clupeine specimen consists of a mixture of molecules with N-terminal alanine and proline. In that paper the molar ratio of N-terminal proline to alanine was estimated, based on the *R* value of the DNP-clupeine or on the amounts of DNP-alanine and *2*, *4*-dinitrophenol, both recovered after acid hydrolysis of DNP-clupeine (*2*). The present isolation of both DNP-prolyl and DNP-alanyl peptides from the tryptic digest of DNP-clupeine, further confirmed the results of the previous reports. As described in the section of results, the molar ratio of N-terminal proline to alanine, calculated from the amounts of DNP-prolyl and DNP-alanyl peptides actually isolated showed rather good agreement with that reported in the previous paper. In every case, the value of the molar fraction of N-terminal proline calculated from the amount of isolated DNP-peptides was found to be somewhat lower than the value estimated from the *R* value of DNP-clupeine. This may be ascribed to the lesser stability of DNP-prolyl peptides.

Some interesting facts found during these experiments will be added. When washed with *N* hydrochloric acid, DNP-prolyl peptides (DNP-Pro·Arg and DNP-Pro·Arg·Arg) were less firmly adsorbed by talc than the corresponding DNP-alanyl peptides, and the former were more easily eluted than the latter from the talc column by running through 1 : 4 (*v*/*v*) ethanol-*N* hydrochloric acid. DNP-Alanyl peptides could be rapidly eluted by 4 : 1 (*v*/*v*) ethanol-*N* hydrochloric acid. Examination with DNP-derivatives of twenty-two peptides containing arginine, which were isolated from a tryptic digest of clupeine (*5*), has shown, that such a property seems to be characteristic of DNP-prolyl peptides including DNP-Pro·Arg, DNP-Pro·Arg·Arg and DNP-Pro·Val·Arg. In the case of DNP-Arg·Pro·Arg, however, no such a property was observed. It has also been shown that a paper electrophoretic technique is of use for the identification and group separation of certain kinds of DNP-derivatives (see Fig. 5).

SUMMARY

1. N-Terminal sequences of two protamines, salmine from salmon (*Oncorhynchus keta*) and clupeine from herring (*Clupea pallasii*), were studied. For that purpose, DNP-protamines were hydrolyzed by trypsin and the DNP-peptides in the dried digests were extracted with methanol. The methanol-soluble DNP-peptides were fractionated into two parts by continuous paper electrophoresis, one (designated as DNP-band A) with one arginine residue and another (DNP-band B) with two. Each of the DNP-bands A and B was purified and fractionated by running through a talc column and

the structure of the isolated DNP-peptides was determined.

2. Since only two DNP-peptides, DNP-Pro·Arg and DNP-Pro·Arg·Arg, were obtained from DNP-salmine, the N-terminal sequence of salmine was inferred to be exclusively Pro·Arg·Arg···.

3. On the other hand, four DNP-peptides, DNP-Ala·Arg, DNP-Pro·Arg, DNP-Ala·Arg·Arg and DNP-Pro·Arg·Arg, were isolated from DNP-clupeine, thus the N-terminal structure of clupeine was concluded to consist of two separate chains, Ala·Arg·Arg··· and Pro·Arg·Arg···. The mixed ratio of the both chains was also estimated.

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CONNECTIVE TISSUE GROWTH IN ALGINIC ACID-GRANULOMA OF RATS

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Recently, biochemical studies of connective tissue formation have been intensively carried out. Dunphy and Udupa (*1*), Taylor and Saunders (*2*) reported the biochemical and histochemical studies on the formation of ground substances and collagen fibers in the healing wound.

Robertson and Schwartz (*3*) found that relatively large amounts of connective tissues were formed locally following the subcutaneous injection of carrageenin (*Irish moss*) into guinea pigs. Their finding offered the convenient method of studying the formation of collagen and acid mucopolysaccharide in the course of connective tissue growth, because by this method it is quite easy to obtain a sufficient large amount of homogenious connective tissue mass for the biochemical analysis.

Jackson (*4*, *5*) made studies on the mechanism of the formation and breakdown of connective tissue fiber in this carrageenin-granuloma. At the group discussion of the symposium on "Connective Tissue" (*6*), he suggested that alginic acid also might induce the granuloma formation in the same way as carrageenin and added that it is, however, not yet known whether alginic acid would promote the reabsorption of once formed collagen fibers. But the detailed biochemical data on this alginic acid-granuloma are not yet published by him.

The present authors, in order to obtain the connective tissue mass of which reabsorption is rather not remarkable, investigated the process of connective tissue growth in granuloma induced by the subcutaneous injection of alginic acid into rats.

METHODS

Preparation of Granuloma—2 ml. of 1 per cent sodium alginate in saline solution were injected into two places on the back of adult male rats. And then the experimental animals were fed on ascorbic acid deficient diet and divided into two groups: one group received the intraperitoneal injection of 10 mg. ascorbic acid per 100 g. body weight on every other day for the first two weeks and another group served as control. As the rat is able to synthesize ascorbic acid within the body, it is not suitable to see a remarkable effect of this vitamin on connective tissue formation. However, this experiment was carried out in order to inspect whether or not the administration of ascorbic acid would accelerate detectably the formation of connective tissue fibers.

Some days after the injection of alginate, the animals were killed and the masses of

newly formed connective tissues were exercised for analysis.

Estimation of Collagen and Mucopolysaccharide Content—Since it is generally recognized that the hydroxyproline content in tissue is parallel with that of collagen, the content of hydroxyproline was estimated by the Neuman and Logan method (*7*). Hexosamine, the component of mucopolysaccharide, was quantitatively determined by the Boas' modification (*8*) of Elson and Morgan method.

Separation and Identification of Hexosamine—It was carried out by the liquid column chromatography on the ion-exchange resin according to the method of Gardell (*9*). The effluent was collected in 1 ml. fractions of which hexosamine contents were estimated by the Boas method. In the concentration-effluent curves, the first peak corresponded to glucosamine and the second to galactosamine.

Fractionation of Collagen—The collagen in granuloma was fractionated by the Jackson method into the neutral-salt soluble (0.2 *M* NaCl), citrate-soluble (1/10 *M* citrate buffer, pH 3.8) and insoluble collagen. Jackson *et al.* found that these three collagen fractions were derived from tropocollagen, reticulin and insoluble collagen fiber, respectively. The hydroxyproline and hexosamine contents in each fraction were determined as above.

Ratio of Hydroxyproline to Total Imino Acid in Collagen—In order to separate the collagen from other tissue proteins, the autoclave-extraction method of Lowry *et al.* (*10*) and also the hot TCA-extraction method of Harkness *et al.* (*11*) were used. About the collagen extracted by these methods, the ratio of hydroxyproline to total imino acid was determined. The content of imino acid was estimated by the Troll method (*12*).

RESULTS

A few days after the injection of alginate, the exposed mass appeared as a transparent viscous jelly and its weight was 2.1–1.3 g. The histological assay (*13*) indicated that the rejuvenated vessels, fibroblasts and collagen fibers were present only in the peripheral region of mass.

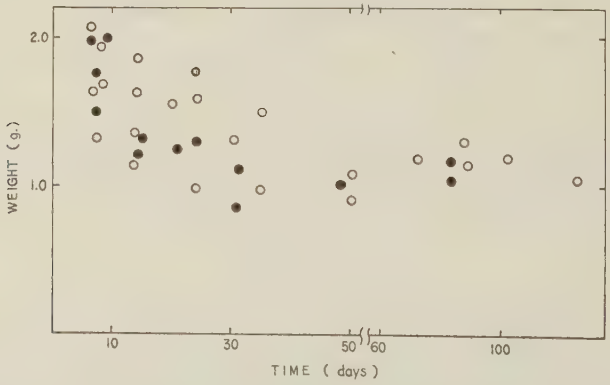

FIG. 1. Weight of granuloma. (○) with and (●) without ascorbic acid administration. Similar symbols were used in all figures.

On succeeding days, the tissue seemed to contract slightly and to become firm. At 20-30 days, the mass had an appearance of a fairly fibrous tissue and its weight was 1.0–1.5 g. Histologically, the collagen fibers increased even

in its central region, separating the alginate jelly into small sections and proliferating around each section. Even after 100 days, there remained a little amount of alginic acid in the mass, in which the connective tissue development continued.

The hydroxyproline content increased quite slowly throughout the experimental period. In an early stage, it was fairly higher in the case of ascorbic acid administration than the control. But at later stage a recognizable difference was not observed. On the histological observation, it was also found that in the early stage the formation of collagen fibers was distinctly promoted by the administration of ascorbic acid.

The hexosamine content increased gradually in an early stage and then decreased slightly. There was no remarkable difference of the hexosamine content between those with and without the ascorbic acid administration.

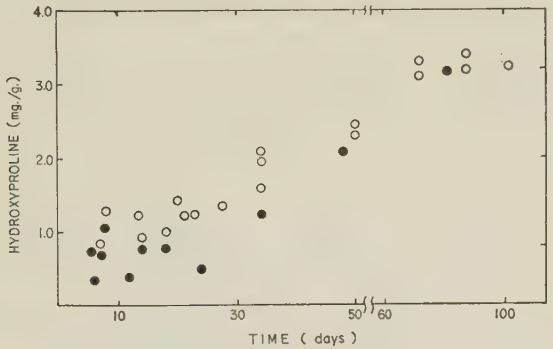

FIG. 2. Hydroxyproline content.

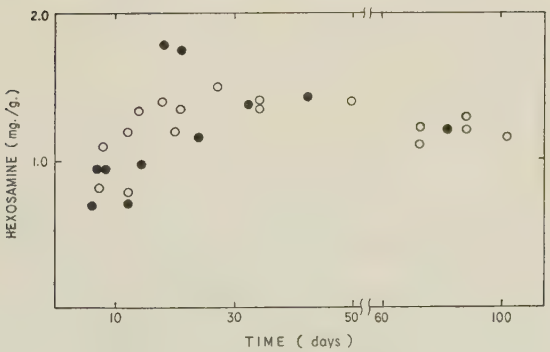

FIG. 3. Hexosamine content.

Fig. 4. shows the relative amount of glucosamine and galactosamine on the column chromatogram (Each figure shows the average value of three cases). The glucosamine content was considerably higher than that of galactosamine. This fact shows that mucopolysaccharides produced in the course of granuloma

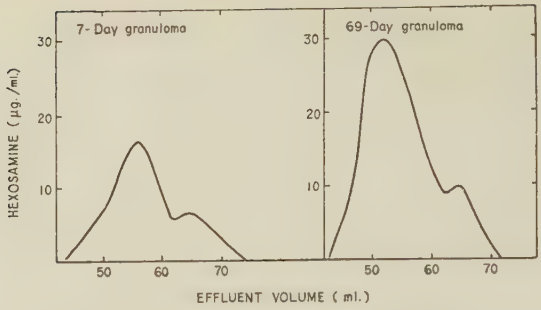

FIG. 4. Chromatogram of amino sugar.

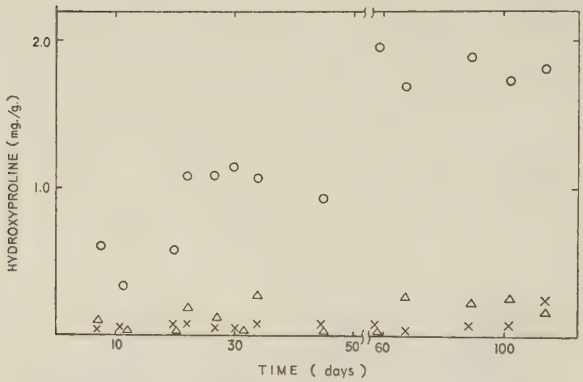

FIG. 5. Hydroxyproline content in neutral salt-soluble (×), citrate-soluble (△) and insoluble collagen fraction (○).

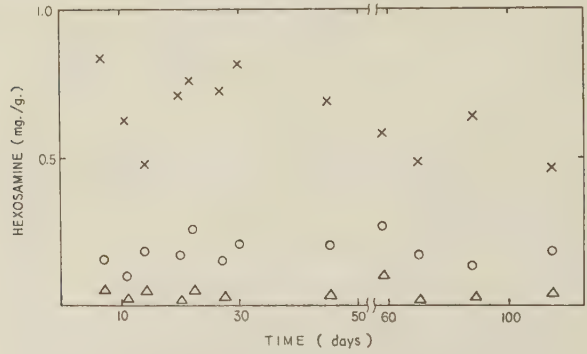

FIG. 6. Hexosamine content in each collagen fraction.

formation contain mainly glucosamine but not galactosamine as their amino sugar components.

Among the three fractions of neutral salt-soluble, citrate-soluble and insoluble collagen, most of hydroxyproline was found in the last fraction. And it was indeed this insoluble collagen which increased in parallel with connec-

tive tissue growth, while the collagen contents of other fractions were unchanged.

Hexosamine was largely found in the neutral salt-soluble fraction and the ratio of hexosamine content in this fraction to that in the insoluble frac-

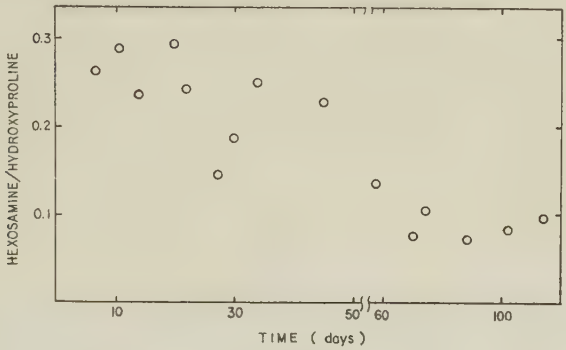

FIG. 7. Ratio of hexosamine to hydroxyproline in insoluble fraction.

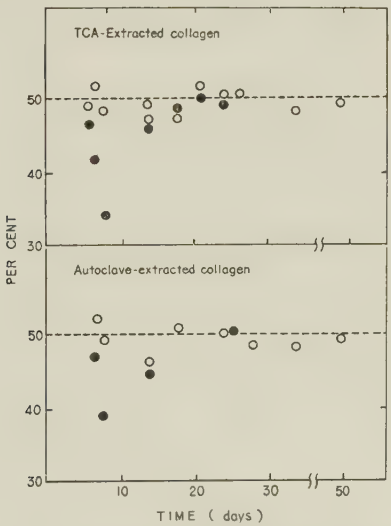

FIG. 8. Ratio of hydroxyproline to imino acid in collagen.

tion decreased gradually, *i.e.* the mucopolysaccharides became insoluble with time.

The ratio of hexosamine to hydroxyproline in the insoluble fraction decreased considerably with the lapse of days.

As shown in Fig. 8, the ratio of hydroxyproline to total imino acid in collagen, extracted by the autoclave-extraction method and also the hot TCA-extraction method, was normally 0.45–0.55. But its ratio, in an early stage without ascorbic acid administration, was considerably lower than the control.

DISCUSSION

In contrary to carrageenin-granuloma, the connective tissue growth of alginic acid-granuloma advanced very slowly and the reabsorption of collagen fibers was scarcely recognized at least for 100 days' period. It is generally known that the rate of degradation of the once formed collagen fibers is quite slight. The remarkable reabsorption of collagen fibers in carrageenin-granuloma may be an abnormal phenomenon. The alginic acid-granuloma might be, therefore, more useful for studying the stable newly-formed connective tissue fibers.

In this present experiment, it was found that the ratio of hexosamine to hydroxyproline in an insoluble collagen fraction decreased considerably with the lapse of days.

About the histochemical observation on the alteration of nature of fibers with their maturing or aging, many reports have yet been published. Jackson (*14*) reported a descriptive analysis of the formation of collagen material in the metatarsal tendon of fowl embryos at different stages of development. According to his histological observations, the recognizable collagen fibers seen in the embryo of early stage are positive in periodic acid-Schiff reaction, but this reaction gradually weakens as bundles of collagen are formed. At a slightly early stage the metachromatic substances are formed to lie in the small interfibriller spaces: the intensity of this stain increases as these spaces enlarge. And, at a later stage when collagen fibers can be distinguished lying in this ground substance metachromasia is gradually lost.

In the healing wound, too, similar phenomenon is generally recognized. Taylor and Saunders discussed about this problem as follows: The disappearance of the metachromasy-stainable intercellular material could have a number of chemical explanations. The material might have been resorbed or altered so that it no longer reacted with the stains; for instance, the acidic groups upon which the staining reactions depend may have become hidden internally during the process of polymerization of mucopolysaccharides. More likely, however, the acidic groups may have become bound by basic groups of tissue origin—probable collagen—and would thus be unavailable for the staining reaction.

From the authors' result, it seems that relative amount of amino polysaccharides associated with the developing collagen fibers decreases with maturing or aging of fibers.

And it was found in this experiment that the collagen formation in an early stage was considerably accelerated by the ascorbic acid administration. The ratio of hydroxyproline to imino acid (sum of hydroxyproline and proline) in collagen, without the administration of ascorbic acid in an early stage, was fairly lower than that with ascorbic acid.

A similar result was reported by Gould and Woessner (*15*). According to their report about the repaired tissue of ascorbic acid deficient guinea pigs, excess proline and glycine refer to the non-collagenous proline and

glycine calculated on the basis that collagen contains 13.4 per cent hydroxyproline, 16.75 per cent proline and 26.8 per cent glycine, and that the apparent collagen is calculated from the hydroxyproline value by multiplying the factor 7.64 (100/13.4). Under the condition of ascorbic acid deficiency, there may be an accumulation of a more primitive precursor than the so-called procollagen or tropocollagen, having excess proline and glycine which is easily converted to a collagen precursor rich in hydroxyproline upon the administration of ascorbic acid.

Anyway, during the development of new connective tissue turns up, the oxidation of proline to hydroxyproline, namely the collagen formation, is fairly retarded when the administration of a sufficient amount of ascorbic acid is omitted.

SUMMARY

The process of connective tissue growth in granuloma induced by the subcutaneous injection of alginate into rats was investigated over 100 day period.

Differently from the carrageenin-granuloma reported by Jackson *et al.*, the development of new connective tissue was quite slowly achieved and the reabsorption of collagen fibers was scarcely recognized throughout the experimental period.

The relative amount of aminopolysaccharides associated with developing collagen fibers decreased with maturing or aging of fibers.

The administration of ascorbic acid accelerated considerably the reaction from proline to hydroxyproline, *i.e.* the formation of collagen fibers.

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1-ALKOXYNICOTINAMIDES AS MODEL COMPOUNDS OF PYRIDINE COENZYMES*

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Addition of nucleophilic reagents such as cyanide (*1*), dithionite (*2*), mercaptans (*3*), imidazols (*4*), ketones (*5*) etc. to pyridine coenzymes has been studied for the elucidation of dehydrogenase mechanism. Although addition on pyridinium ring may take place at 2-, 4-, or 6-position, only 4-position of DPN** is attacked by cyanide (*6*) or dithionite (*7*) as shown by experiments with deuterium. As yet little work has been made in order to estimate the condition favoring addition at 4-position. Chemical behavior of model compounds has been studied with 1-substituent of alkyl (*8*), benzyl (*9*) or benzoyl group (*10*), but the site of addition was not determined in the case of cyanide (*11*). Moreover, these model compounds have 1-substituent of weaker inductive effect than those of pyridine coenzymes. Recently, T. Okamoto and H. Tani succeeded for the first time in the direct introduction of cyanide in pyridines through quaternary salts of pyridine 1-oxides (*12*).

In the present work, 1-alkoxynicotinamides were synthesized, and their behavior to nucleophilic reagents was examined. As the addition products have light absorption at 380 mμ, the reaction was followed spectrophotometrically. Taking advantage of the spontaneous decomposition of the addition product into cyanonicotinamides, the site of addition was determined to be at 4- and 6-positions. The cause of selective addition at 4-position and the specific light absorption of pyridine coenzymes were discussed.

EXPERIMENTALS

Pyridine Carboxylic Acid Esters and Amides—Ethyl nicotinate (b.p. 87°, 8 mm. Hg.) and nicotinamide (m.p. 130°) were synthesized from coal tar base quinoline by Se oxidation (*13*), dimethyl quinolinate (m.p. 54°) and diamide (m.p. 209°), from quinoline by oxidation with $KMnO_4$ and $CaCO_3$, dimethyl cinchomeronate (picrate, m.p. 129°) and diamide (m.p. 175° decomp.), from cinchonine by oxidation with fuming HNO_3, dimethyl isocinchomeronate (m.p. 163°) and diamide (m.p. 310° decomp.), from 2-methyl-5-ethylpyridine (b.p. 178°) by oxidation with $KMnO_4$. Esterification was performed with concentrated H_2SO_4, ammonolysis with NH_3 in methanol or water, in all cases. Ethyl 6-methylnicotinate

* This paper was presented at the General Meeting of Japan Pharmaceutical Society held in June 1959, at Tokyo.

** Diphosphopyridine Nucleotide is abbreviated as DPN.

was kindly given by Mr. H. Tani. Dimethyl 6-methylcinchomeronate (m.p. 70°) was synthesized by cyanide addition reaction to ethyl-1-methoxy-6-methylnicotinate, then and hydrolysis esterification with the same method described in this report.

Other Materials—KCN used was 97.1 per cent pure by titration with $AgNO_3$. Alumina was reactivated at 400° for 6 hours.

Instruments—Infrared spectra were recorded with Kaken Model DS-301 double-beam recording spectrophotometer equipped with NaCl prism. Solid samples were pressed into disks using KBr as the diluent. Ultraviolet spectra were recorded with Beckman Model DK-2 recording spectrophotometer and Hitachi Model EPU-2 spectrophotometer. pH values were measured by Toa Dempa Model HM-5 pH-meter with a glass electrode.

Synthesis of Model Compounds

Nicotinamide 1-Oxide—Nicotinamide (10.0 g.) was dissolved in glacial acetic acid (105 g.) and H_2O_2 (35 per cent, 10 g. at first, another 10 g. 3 hours later), and the mixture was kept at 70° for 7 hours. Solvent was removed under a reduced pressure at 70°. During evaporation water was added several times to avoid explosion. The remaining white powder was recrystallized from hot water. Yield was 7.8 g. or 72 per cent of the theoretical. White needles were insoluble in ether and alcohols, soluble in acids and gave m.p. 283° decomp. (reported, 283° (*14*), 293° (*15*)).

Infrared spectra: Main absorption, 3500–3200, 1690, 1630, 1243, 1160 cm^{-1}. Strong absorption at 1243 cm^{-1} suggests the presence of N-oxide.

Ultraviolet spectra: Maximum absorption at 270 mμ (11,900)
Minimum absorption at 242 mμ (3,500) in absolute methanol at 15°

Elementary analysis: Found C, 52.01 H, 4.32 N, 20.5
Cald. for $C_6H_6O_2N_2$ C, 52.17 H, 4.38 N, 20.3

Paper chromatography: R_f 0.44 with 80 per cent acetone. (Nicotinamide: R_f 0.80 in the same condition).

Other N-Oxides—Ethyl nicotinate 1-oxide (m.p. 103°), ethyl 6-methylnicotinate 1-oxide (m.p. 46–48°) and dimethyl 6-methylcinchomeronate 1-oxide (m.p. 115°) are obtained by essentially the same method as described above.

1-Methoxynicotinamide Methylsulfate (*1-Methoxy-3-carbamoyl-pyridinium Methylsulfate*)—A suspension of nicotinamide 1-oxide (2.5 g. dried) in dimethyl sulfate (10 g., freshly distilled) was shaken vigorously with exclusion of moisture at 100° for 30 minutes. After the reaction, the mixture was separated into two clear layers and kept at room temperature for 3 days in a desiccator. Colorless needles in the lower layer were collected and washed carefully with ether (dried with Na metal). Yield was 3.5 g. or 73 per cent of the theoretical. This highly hygroscopic crystals can be recrystallized from dry acetone m.p. 92°. Soluble in water, methanol ethanol and insoluble in ether. Stable in methanol for a month. As the isolation of this compound from water is difficult, its picrate was prepared by treatment with sodium picrate and was recrystallized from hot methanol (m.p. 162°).

Infrared spectra: Main absorption at 3400–3000, 1690, 1600, 1630, 1450, 1200–1300, 1030, and 750 cm.$^{-1}$ were assigned to amide, pyridine ring, alkyl group, and sulfate.

Ultraviolet spectra: Maximum absorption at 266 mμ (4,500)
Minimum absorption at 247 mμ (2,800) in absolute methanol at 15°.

Elementary analysis: Found C, 37.01 H, 4.78 N, 9.9 S, present
Cald. for $C_8H_{12}O_6N_2S$ C, 36.34 H, 4.58 N, 10.6 S, present

Analysis of the picrate: Found C, 41.09 H, 2.80 N, 18.5
Cald. for $C_{13}H_{11}O_9N_5$ C, 40.95 H, 2.91 N, 18.3

1-Ethoxynicotinamide Ethylsulfate (*1-Ethoxy-3-carbamoyl-pyridinium Ethylsulfate*)—Nicotinamide 1-oxide (dried, 1.5 g.) was suspended in diethyl sulfate (5.0 g.) and treated in the same way as for 1-methoxynicotinamide. However, it required 90 minutes to obtain clear viscous lower layer. The compound was washed with dry ether to remove remaining diethyl sulfate. Yield 3.1 g. or 98 per cent of the theoretical. White waxy crystals thus obtained had m.p. 75–78°, and were highly hygroscopic, soluble in water, methanol, ethanol, and insoluble in ether. The picrate, m.p. 135.5°.

Infrared spectra: Identical with the spectrum of 1-methoxynicotinamide, but a strong absorption at 920 cm^{-1}.

Ultraviolet spectra: Maximum absorption at 265 mμ (4,500)
Minimum absorption at 248 mμ (3,100) in absolute methanol at 15°.

Elementary analysis of the picrate:

Found	C, 42,65	H, 3.20	N, 17.5	
Cald. for $C_{14}H_{13}O_9N_5$	C, 42.54	H, 3.31	N, 17.7	

1-Butoxynicotinamide p-Toluenesulfonate (*1-n-butoxy-3-carbamoyl pyridinium p-Toluenesulfonate*)—A mixture of *n*-butyl-*p*-toluenesulfonate (4.2 g., b.p. 145° at 0.5 mm. Hg) and nicotinamide 1-oxide (1.2 g.) was treated in the same way as for 1-methoxynicotinamide, at (100°, 4 hours). After washing with dry ether, the mixture was extracted with water. The residual crystals were found to be nicotinamide 1-oxide (0.75 g. recovered). Ultraviolet spectroscopy of the compound was performed with its aqueous solution.

Other 1-Alkoxynicotinic Acid Derivatives—Ethyl nicotinate 1-oxide and ethyl 6-methyl-nicotinate 1-oxide were alkylated with dimethyl sulfate, diethyl sulfate, and *n*-butyl *p*-toluenesulfonate in the same way as described above. They were reacted at 100° for 2 hours. Crystalline 1-methoxypyridinium iodide (m.p. 98°) was synthesized by essentially the same method as reported by E. Ochiai (*16*). 1-Alkoxynicotinic acid was obtained by acid hydrolysis (1 *N* HCl, at 100°, 4 hours) of 1-alkoxynicotinamide.

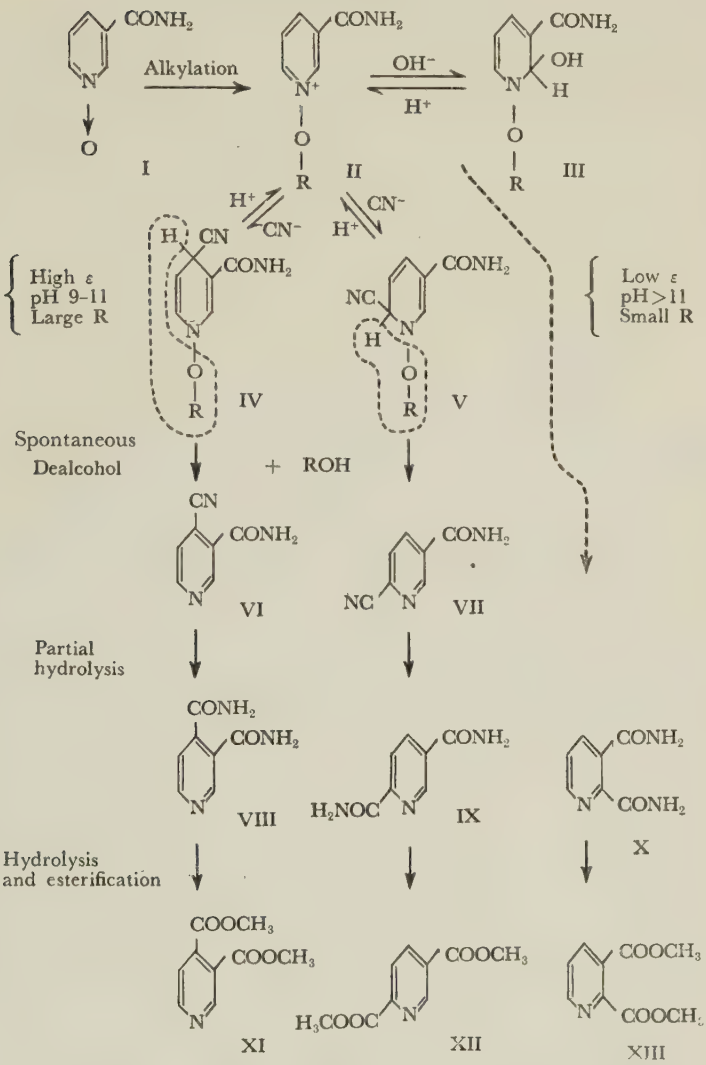
Procedure of Cyanide Addition Reaction—Ten mM of Alkoxynicotinamide (2.64 g. for 1-methoxy-, and 2.92 g. for 1-ethoxy-) were dissolved in 5 ml. of solvent. The solution was dropped into a solution of KCN (20 mM or 1.30 g. in 5 ml. of the same solvent) during 30 seconds. The reaction produced heat, but the temperature must be kept at 10° by cooling in a water bath. Yellow color developed immediately, which turned to dark brown within several minutes. One hour later, the reaction was stopped by addition of sulfuric acid (2 *N*, 5 ml.) and remaining CN^- was removed by bubbling the solution with air. In the study of pH effect, 500 mg. of 1-alkoxynicotinamide was dissolved in water (2 ml.) and dropped into a buf- fered KCN solution (40 ml.). Borate buffer was prepared by adding a certain volume of 0.1 *N* NaOH to 0.1 *N* boric acid (50 ml.) and the total volume was adjusted to 100 ml. To this buffer (30 ml.) 0.4 *M* KCN (10 ml.) was added and pH was adjusted by 1 *N* HCl.

RESULTS

Chemical reactions in the present study are summarized in Diagramm 1.

A. Qualitative Analysis of the Reaction Product of Cyanide Addition 4-Cyanonicotinamide—As no satisfactory technique for separating 4- and 6-isomers was available, a possibility of steering the cyanide addition towards the 4-cyano-isomer was considered. Formation of the latter could arise through increased dielectric constant of the solvent and a large alkoxyl group, as will be shown later in quantitative analysis. The precipitate of the reaction

DIAGRAMM 1
Compounds and reactions studied



I. Nicotinamide 1-oxide, II. 1-Alkoxynicotinamide, R for methyl, ethyl or *n*-butyl group. III. Addition compound of 1-alkoxynicotinamide with OH^-. IV. and V. Addition compound of 1-alkoxynicotinamide with CN^- at 4- and 6-position, respectively. VI. 4-Cyanonicotinamide. VII. 6-Cyanonicotinamide. VIII. Cinchomeronic acid diamide. IX. Isocinchomeronic acid diamide. X. Quinolinic acid diamide. XI. Dimethyl cinchomeronate. XII. Dimethyl isocinchomeronate. XIII. Dimethyl quinolinate. I. → II. Alkylation. II. → III. Hydroxyl ion addition. II. → IV. or V. Cyanide addition. IV. → VI. and V. → VII. Irreversible decomposition of the addition products into alcohol and cyanonicotinamide. VI. → VIII., VII. → IX. Partial hydrolysis during reaction. VIII. → XI., IX. → XII. and X. → XIII. Complete hydrolysis and esterification for quantitative separation and identification of cyanonicotinamides.

mixture of ethoxynicotinamide in water was washed with cold water and extracted with a mixed solvent (methanol 4, dimethylformamide 1). Recrystallization from dimethylformamide gave colorless prisms (m.p. 263° decomp.). Cyanide addition reaction was carried out with the equal procedure to that described above with ethyl-1 ethoxy nicotinate in water. Extraction with $CHCl_3$ and separation on alumina column using C_6H_{14} and $CHCl_3$ yielded ethyl 4-cyanonicotinate (m.p. 53°). Ammonolysis with methanolic ammonia gave white crystals, m.p. 263° decomp. and mixed m.p. with the former 263° decomp. The structure was determined by hydrolysis and esterification. The resulting dimethyl cinchomeronate was crystalized as picrate (m.p. 129°). Mixed m.p. with authentic sample was 129°.

Infrared spectra: Main absorption at 3500–3000, 2250, 1680, 1620 $cm.^{-1}$ were assigned to amide, nitrile, and pyridine ring.

Elementary analysis: Found C, 57.37 H, 3.29 N, 29.1
Cald. for $C_7H_5ON_3$ C, 57.09 H, 3.43 N, 28.7

6-Cyanonicotinamide—The compound was prepared from the reaction mixture of 1-methoxynicotinamide in 50 per cent dioxane by the same method as used for 4-cyano isomer (m.p. 247° decomp.). 6-Cyanonicotinamide (m.p. 273° decomp. reported 275°–277° decomp. (*17*)) was also obtained by ammonolysis of ethyl 6-cyanonicotinate (m.p. 55.5°). Hydrolysis and esterification of the former gave dimethyl isocinchomeronate (m.p. 163°). Infrared spectrum of 6-cyanonicotinamide was almost identical with that of 4-cyano isomer.

Isocinchomeronic Acid Diamide—An extremely insoluble residue (m.p. 305° decomp.) of the precipitate from the reaction mixture was recrystallized from boiling nitrobenzene (m.p. 310° decomp.). Admixture of this product with isocinchomeronic acid diamide showed m.p. 310° decomp.

Dimethyl Quinolinate—The reaction mixture of 1-methoxynicotinamide in 50 per cent dioxane was hydrolyzed, esterified and chromatographed as will be described later. The mother liquor of dimethyl cinchromeronate picrate was paperchromatographed with hexane and detected under ultraviolet light. One spot with R_f value of 0.15 which corresponded to that of dimethyl quinolinate was eluted with methanol. The absorption curve of the eluate was found to be the same as that of dimethyl quinolinate (maximum at 263 $m\mu$).

Alcohols—These are detected through xanthogenic acid.

B. Quantitative Analysis of the Addition Products

Complete hydrolysis and esterification of the reaction mixture was necessary for this purpose owing to the partial hydrolysis and low solubility of cyanonicotinamides. To the reaction mixture 20 per cent KOH (10 ml.) was added, heated at 100° for 5 hours, neutralized, and dried *in vacuo*. This was esterified with conc. H_2SO_4 (20 ml.) and methanol (15 ml.) at 100° for 2 hours. The reaction mixture was poured on ice (100 g.) Na_2CO_3 (60 g.) and extracted with ether (100 ml., 4 times). After ether was removed, the product was chromatographed on a column of alumina (1.8 $cm.^2 \times 15$ cm.). Elution of the column with bezene and hexane (1 : 1) gave dimethyl cinchomeronate, elution

with ether and benzene (1 : 1) gave dimethyl isocinchomeronate, and with ether and methanol (1 : 1) gave remaining tar. Dimethyl cinchomeronate was weighed as its picrate recrystallized from methanol. The ratio of 4-cyano- to 6-cyanonicotinamide was obtained by the ratio of weight of dimethyl cinchomeronate to that of dimethyl isocinchomeronate. The same procedure was adopted for diamide of quinolinate, cinchomeronate, and isocinchomeronate which resulted in recovery of 77.2 per cent, 79.6 per cent and 78.2 per cent, respectively. Results are shown in Tables I and II. In the case of reaction at pH 9.2 and pH 14, 1-alkoxynicotinamides were recovered and identified as picrate. Increased dielectric constant, lowered pH of the solvent, and ethoxyl group on the compounds favored addition at 4-position.

Table I

Solvent Effect on the Ratios of 4- and 6-Position Cyanide Addition

	1-Methoxynicotinamide		1-Ethoxynicotinamide	
Solvent	Total Yield[1]	Ratio 6/4[2]	Total Yield[1]	Ratio 6/4[2]
100% Water	43.1%	0.24	38.8%	0.07
50% Methanol	45.5%	0.92	40.6%	0.51
100% Methanol[3]	37.0%	1.35	45.9%	0.91
50% Dioxane	45.5%	2.63	54.3%	0.96

1) Total yield: Percentage of theoretical yield of dimethyl pyridinedicarboxylate from 1-alkoxynicotinamides.

2) The ratio was obtained by dividing the weight of dimethyl isocinchomeronate by the weight of dimethyl cinchomeronate calculated from the weight of dimethyl cinchomeronate picrate.

3) KCN was used as suspension.

Table II

pH Effect on the Ratio of 4- and 6-Position Cyanide Addition

	1-Methoxynicotinamide		1-Ethoxynicotinamide	
pH	Total Yield[1]	Ratio 6/4[1]	Total Yield[1]	Ratio 6/4[1]
9.2	0 %[2]	—	0 %[2]	—
10.0	37.2%	0	25.1%	0
10.8	40.2%	0.13	45.4%	0
11.6	42.5%	0.32	50.0%	0.09
14	0 %[2]	—	0 %[2]	—

1) Total yield and ratio is calculated in the same way as in Table I.

2) Alkoxynicotinamide was recovered as picrate after complete removal of CN^-.

C. Ultraviolet Spectroscopy

A remarkable lihgt absorption at 380 mμ was observed in the addition

reaction, and the absorption vanished within several minutes in case of cyanide Fig. 1. This phenomenon was analyzed in the following order.

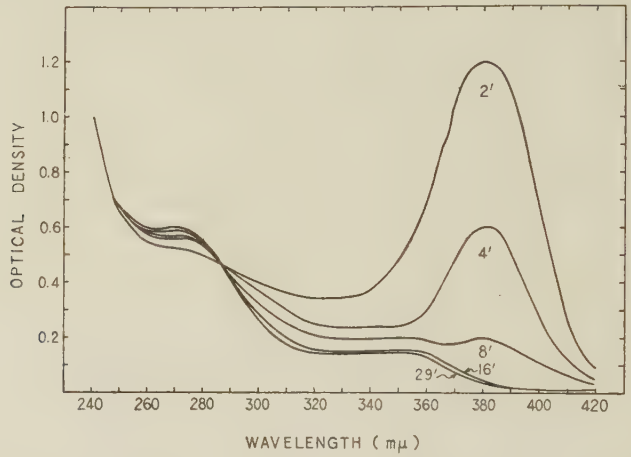


FIG. 1. Cyanide addition reaction followed by the recording spectrophotometer.

Final concentration in cuvette: KCN 2 *M*, 1-methoxynicotinamide methylsulfate 10^{-4} *M*. Solvent: 10 per cent methanol in water. 13°. Speed of scanning: 2 minutes.

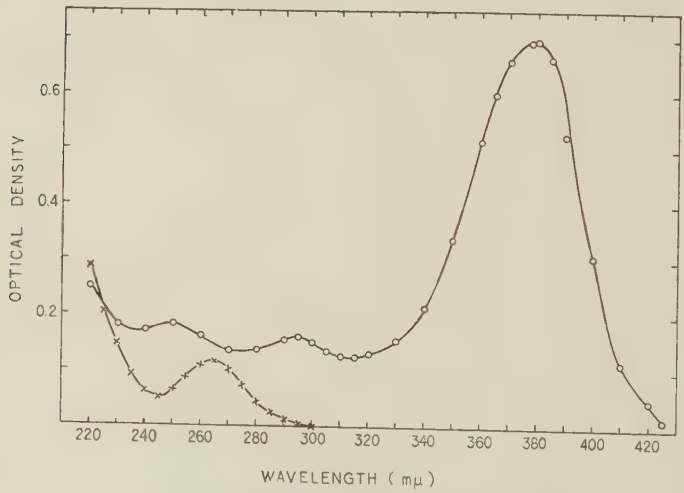


FIG. 2. Absorption spectrum of 1-methoxy nicotinamide.

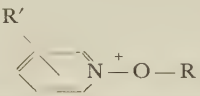
—×—, in neutral aqueous solution (pH 8.0); —○—, in alkaline aqueous solution (pH 11.6). 2.5×10^{-5} *M* in both case, at 15°. Methylsulfate was used.

Effect of pH on Absorption Curve—1-Alkoxynicotinamide has no light absorption in longer wave-length region than 300 mμ in neutral solution but

shows strong absorption at 380 mμ in alkaline solution (Fig. 2). The effect of cations such as K^+, Na^+ and NH_4^+ is the same if pH values are equal.

TABLE III

Absorption Maxima of 1-Alkoxypyridinium Compounds in Alkali



R \ R′	H	3-COO⁻	3-CONH₂	3-COOEt	3-COOEt, 6-CH₃
CH_3	(257)	373	380	378	397
C_2H_5	—	373	380	378	397
n-C_4H_9	—	—	380	380	397

In water, pH 11.7. Et means C_2H_5.

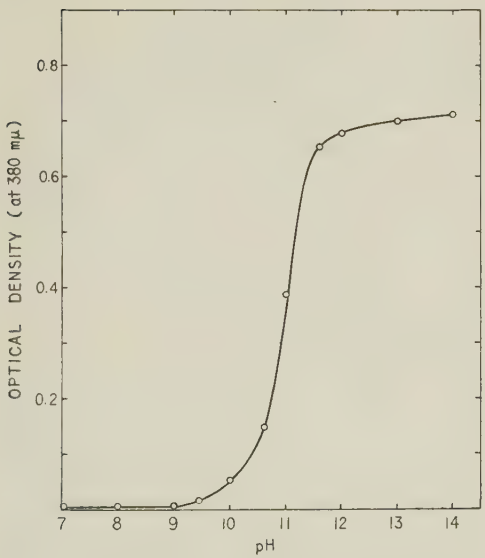


FIG. 3. Effect of pH on the absorption at 380 mμ. 1-methoxynicotinamide methylsulfate in borate buffer. The position of absorption maximum was constant at any pH.

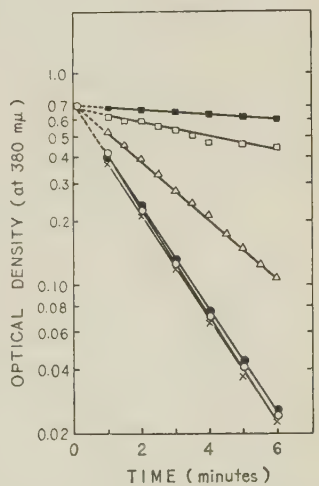


FIG. 4. Effect of KCN concentration on the reaction velocity. —■— 1/1000 N, —□— 1/100 N, —△— 1/10 N, —●— 1/4N, —○— 1/2 N, —×— 1 N, KCN concentration. pH 11.6 with Na_2CO_3, 1-methoxynicotinamide methylsulfate 2.5×10^{-5} M 16°.

The minor maxima were observed at 250 mμ and 295 mμ both with 1-methoxy- and 1-ethoxynicotinamides, but with 1-butoxy derivatives the minor peak was found at 253 mμ owing to *p*-toluenesulfonate. In order to determine

the specificity of the absorption, several kinds of 1-alkoxypyridinium compounds were studied (Table III). Though cyanide is directry introduced into

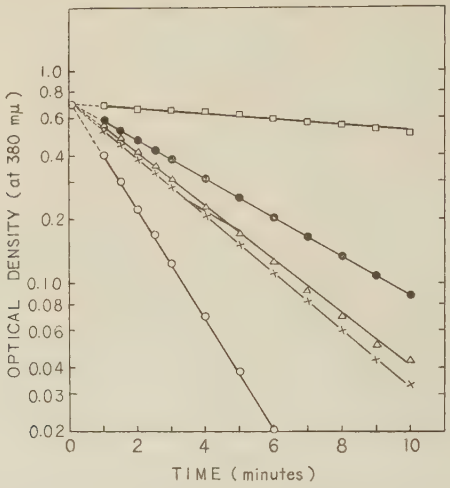


FIG. 5. Effect of solvent on the reaction velocity.
—□— 50 per cent dioxane, KCN 1 *M*, —×— 50 per cent methanol, KCN 1 *M*, —○— 100 per cent water, KCN 1 *M*, —△— 50 per cent methanol, KCN 1/2 *M*, —●— 50 per cent ethanol, KCN 1/2 *M*.
1-Methoxynicotinamide methylsulfate 2.5×10^{-5} *M*. pH 11.6 by Na_2CO_3. 16°.

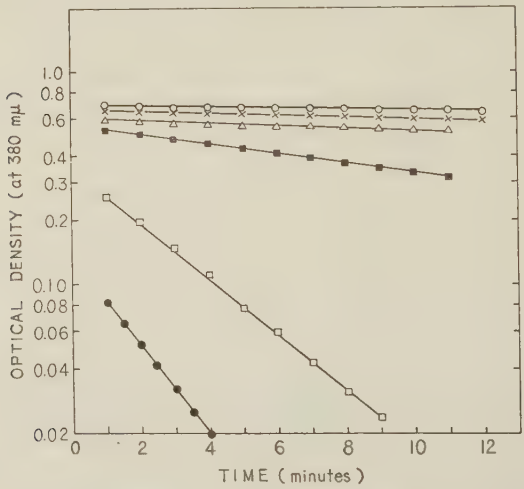


FIG. 6. Effect of pH on the reaction velocity.
—○— pH 14, —×— pH 13, —△— pH 12, —■— pH 11.5, —□— pH 11.0, —●— pH 10.5, Final concentration of KCN was 1/10 *N* and that of 1-methoxynicotinamide methylsulfate was 2.5×10^{-5} *M* in water. 0.1 *N* borate buffer. pH is adjusted by *N* NaOH or *N* HCl after KNC is added in the buffer.

pyridine, no absorption near 380 mμ is observed. Methyl group at 6-position

has some bathochromic effect, but 1-alkoxy groups show no marked difference among them, and intensity was nearly the same in methoxyl (28,000) and ethoxyl (30,000) at pH 11.7. Absorption at 380 mμ was greatly influenced by pH value (Fig. 3) but the maximum lies near 380 mμ in every case. An attempt was made to determine the relation between the site of addition and absorption, by means of blocking 2, 4, or 6-position of 3-carbonyl pyridinium compounds such as dimethyl 1-alkoxy-6-methyl cinchomeronate. However, synthesis of 1-alkoxy derivatives of pyridine-polycarboxylic acid was not successful.

Effect of KCN Concentration on the Reaction Velocity—Straight line is produced when the logarithm of the optical density is plotted against time (Fig. 4). The slope of the line becomes steeper with the increase in concentration of KCN added and finally it reached to a maximum. This suggests the appearance of other rate limiting process than cyanide addition.

Effect of Solvent on the Reaction Velocity—The concentration of solvents was limited by the solubility of KCN in them. If the reaction was started by addition of the material into KCN solution only weak absorption was observed in case of low dielectric constant. The reaction was retarded in solution of low dielectric constant (Fig. 5).

Effect of pH on the Reaction Velocity—The reaction was started by adding material solution into pH adjusted KCN solution. It was observed that the reaction was retarded in strong alkali (Fig. 6). This suggests the presence of equilibrium between compounds II and III shown in Diagramm 1. Absorption at 380 mμ was observed immediatly after the material was added into aqueous alkaline solution, but gradual increase of the absorption was observed in methanolic NaOH solution. These results shown in Figs. 2 to 7 are essentially valid for other 1-alkoxynicotinic acid derivatives indicated in Table III.

DISCUSSION

The Light Absorption at 380 mμ—The absorption appears whenever 1-alkoxy-3-carbonylpyridinium compounds are brought into alkaline solution. Although other anionoid agents may attack the pyridinium ring, the absorption is not distinguished from one caused by hydroxyl ion as the addition takes place only in alkaline solution. In the case of cyanide the absorption decreases spontaneously. It is likely that the ease of hydroxyl ion addition is caused by inductive effect of alkoxyl group. This would be evidenced by the fact that ethyl 1-methyl-nicotinate and 1-methylnicotinic acid form cyanide addition complex only in alcoholic solution (*8*), while 1-alkoxy-3-caronyl compound does so even in aqueous solution. It is not clear whether every cyanide addition complex (formulae IV and V in Diagramm 1) has the same absorption or not, but it seems quite possible as DPN shows an absorption near 340 mμ with any kind of nucleophilic agent. 1-Methyl-cyanodihydronicotinamide with absorption at 340 mμ was also isolated (*11*). The specific absorption of reduced pyridine coenzymes is attributed to the merocyanine dye like conjugated system including carbonyl group and pyridine ring (*18*).

The bathochromic and hyperchromic nature of this addition complex as compared with that of pyridine coenzymes and 1-alkylnicotinamide must be caused by the lone pairs of the alkoxy oxygen in the conjugated system. The other evidence of a longer conjugated system may be obtained with dimethyl 1,4-dihydro-2,6-dimethyldinicotinate which has the maximum absorption at 380 mμ (*19*). There is no reason to assume that only addition complex at 4-position has the absorption around 380 mμ, because ortho-dihydro derivative of 1-alkylnicotinamide with absorption at 350 mμ was isolated (*20*), and DPN reduced with $NaBH_4$ retains the absorption at 340 mμ after enzymatic reoxidation (*21*). Addition of hydroxyl ion (formula III in Diagramm) may take place at ortho-position and this was inferred from data on DPN (*22*) and pyridone formation (*23*), (*24*).

On the Reaction Condition and Ratio of 4- and 6-Addition—It is confirmed that 4-position additions is favored by a high dielectric constant, a lower pH value of the solvent, and a large alkoxyl group. Increased addition at para-position and absence of addition product at 2-position by a large *N*-substituent would suggest the perticipation of steric hindrance in addition reaction. Steric hindrance of alkoxyl group against ortho-position of pyridium ring may be smaller than that of alkyl group. Ortho-position addition has not been reported with DPN and 1-alkylpyridinium compounds except the cases of hydroxyl (*22*), (*23*) or hydride ion attack (*20*), (*21*), where these anions are small. The decreased reaction velocity in low dielectric constant can be explained by the fact that cyanide addition product (Diagramm 1 compounds IV and V) have no ionized structure and are stable if the transition states are ionized (*8*) (*25*). In contrary to the idea that high pH may accelerate the anionoid addition, the reaction velocity is remakably lowered in strong alkaline solution. This effect suggests the presence of equilibrium between 1-alkoxypyridinium and the hydroxyl addition compound (Diagramm 1 compound III). The presence of a limit in the effect of cyanide concentration on the reaction velocity suggests the presence of another rate determining step (Diagramm 1 compounds IV to VI, V to VII or III to II). As the logarithmic plot of the optical density change against time was found to be straight, the reaction appears to be a first order to free 1-alkoxynicotinamide, and in case of extremely high KCN concentration, to some addition complex.

Although it is difficult at present to explain these effects of conditioning factors by electronic theory, it may be significant to induce some clue for specific behavior of pyridine coenzymes, *i.e.* their addition reaction is observed with large *N*-substituents, in aqueous solution, and rather weak alkaline medium, the conditions which favor 4-position addition in these model compounds.

SUMMARY

1. 1-Alkoxynicotinamide and related compounds were synthesized as model compounds of pyridine coenzymes. Cyanide addition reaction to these

compounds was studied and the resulting cyanonicotinamides and their derivatives were isolated.

2. The ratio of 4- and 6-cyanonicotinamide formed was measured by the weight of dimethyl pyridine-dicarboxylates. Addition at 4-position was favored by a high dielectric constant and a rather low pH value of the solvent and model compounds with large 1-substituents.

3. 1-Alkoxy-3-carbonylpyridinium compounds were found to have a marked absorption near 380 mμ in alkaline solution. This absorption vanished during the course of cyanide addition reaction owing to spontaneous decomposition of the cyanide addition product into cyanonicotinamide and alcohol. The rate of disappearance of the absorption in the reaction decreased at a lower KCN concentration, in strong alkali, and at lower dielectric constant.

4. Selective addition at 4-position and light absorption near 340 mμ of pyridine coenzymes which observed in the addition reaction are discussed.

The writer expresses thanks to Prof. T. Okamoto for suggesting this investigation as well as for constant guidance in the course of the work, and to many courtesies extended to him during this work in the Department of Pharmaceutical Chemistry. The writer is indebted to Mr. H. Tani for gift of many samples. Elementary analysis was kindly performed by members of the Central Analysis Department. The writer also expresses his heartful gratitude to Prof. N. Shimazono and Prof. E. Ochiai for their helpful advices and continuous encouragement.

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BIOCHEMICAL STUDIES ON PEARL

VII. FRACTIONATION AND TERMINAL AMINO ACIDS OF CONCHIOLIN

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The mother-of-pearl of molluscan shells and nacre of pearl consist of many extremely thin layers of organic substances and the crystals of calcium carbonate (*1*, *2*). Among these organic substances, conchion, a kind of scleroprotein, is the main constituent, and physical observations on the decalcified shell show that it forms the thin films of lacelike structure (*3*). Though the amino acid composition of conchiolin has been studied by many researchers (*1*, *4*, *5*), its nature and chemical structure have not yet established. This paper is concerned with the results of the purification and its chemical structure of the protein.

Fractionation of conchiolin with suitable reagents was first examined. Though this protein is insoluble in water, in ordinary organic solvents, in weak acids and in weak alkalis, a part of conchiolin was found to be soluble in Schweizer solution and in 70 per cent acetic acid. Solutions of the complex-salts such as copper-ethylenediamine (*6*) and tetrapyridine-cobaltous chloride also proved to be capable of fractionating conchiolin. With the several fractions of conchiolin thus obtained, amino-terminal and carboxy-terminal amino acids were determined. Determination of amino-terminal amino acids was carried out by the DNP-method (*7*) and carboxy-terminal amino acids were determined by the hydrazinolysis method (*8*).

EXPERIMENTAL

Preparation of Conchiolin—The shells obtained from three-years-old Japanese pearl oysters, *Pinctada martensii*, cultured at Matoya Bay in Mie Prefecture, were used for the preparation of conchiolin. The shells were washed to remove the smudges or the scales of acorn barnacles that covered them, and were dipped in approximately 1 *N* hydrochloric acid to decompose calcium carbonate. In a day or two, the greater part of calcium carbonate was decomposed by this treatment and nacreous layers of the shells were then easy to separate from the prismatic layers (outer layers).

After calcium carbonate was completely dissolved, raw insoluble conchiolin remained. 200 g. of raw conchiolin was obtained from 1 kg. of the shell material. It was washed with water repeatedly, then the protein was electrodialyzed against distilled water through cellophane membrane.

Fractionation of Conchiolin—Air-dried conchiolin was pulverized by the agate-mortar and was kneaded with a little quantity of 70 per cent acetic acid. The paste was trans-

ferred into a beaker and was mixed with a large quantity of 70 per cent acetic acid under strong stirring and then centrifuged (2,000 r.p.m. 10 minutes). Soluble component of conchiolin in 70 per cent acetic acid (tentatively named Conchiolin I) was precipitated by the addition of a large quantity of acetone. By repeated dissolution and precipitation, Conchiolin I was purified and air-dried.

Conchiolin I thus prepared was further treated with 2 *N* ammonia and separated into soluble Conchiolin I-1 and insoluble Conchiolin I-2 in ammonia.

Another fractionation of conchiolin was tried by using the following solution such as Schweizer solution, 30 per cent aqueous solution of tetra-pyridine cobaltous chloride, $[Co(py)_4]\ Cl_2$, at pH 6.13, and also 14 per cent copper-ethylenediamine solution of which pH was brought to 4 with dilute acetic acid.

Soluble and insoluble fractions in tetra-pyridine cobaltous chloride were named Conchiolin III and IV, and also soluble and insoluble parts in copper-ethylenediamine solution were named Conchiolin V and VI respectively.

Determination of Free Amino-Terminal Amino Acid Residues of Conchiolins—Free amino-terminal (abbreviated to N-terminal) amino acid residues were determined by the DNP method established by Sanger (*7*); 0.1 g. of conchiolin or conchiolin fraction was mixed with 0.1 g. of sodium bicarbonate in 1 ml. of water. To this mixture, 0.1 ml. of fluoro-2,4-dinitrobenzene (DNFB) dissolved in 2 ml. of ethanol was added. The mixed solution was sealed in a small glass tube and was shaken for two hours at room temperature in a dark place. After the reaction was completed, ethanol was distilled off under reduced pressure. The residue was washed five times with ether and then the unchanged DNFB was removed. The aqueous solution was acidified with a small quantity of 6 *N* hydrochloric acid and the precipitate was washed with water, ethanol and ether successively.

A small amount of DNP-conchiolin thus obtained was sealed in a glass tube with 20 times of 6 *N* hydrochloric acid in volume and hydrolyzed for 10 hours in a Rhoto-Meyer furnace at 110°. From the hydrolysate DNP-amino acids were extracted with 20 ml. of ether five times repeatedly.

The qualitative determination of DNP-amino acids was carried out by paperchromatographic methods (*9*), for which two developing solvent systems were used, one was *tert*-amylalcohol and the other was 10 per cent ethanol in benzylalcohol, both were buffered with equal volume of phthalate buffer solution (*M*/10, pH 6.0)

Determination of Free Carboxyl-Terminal Amino Acid Residues of Conchiolins—Free carboxyl-terminal (abbreviated to C-terminal) amino acids of Conchiolin I-1 were determined by the hydrazinolysis method proposed by Akabori (*8*); 30 mg. of Conchiolin I-1 was treated with 0.5 ml. of anhydrous hydrazine at 100° in a boiling water bath for 5 hours. By this decomposition only C-terminal amino acids were obtained as free amino acids, while other amino acids became amino acid hydrazides. After the reaction was finished, excess of anhydrous hydrazine was removed under reduced pressure by keeping hydrazinate in a sulfuric acid desiccator for 24 hours. To the residue 5 ml. of water was added and pH of the solution was adjusted to 7 with a sodium bicarbonate solution and then 0.3 ml. of isovaleraldehyde was added to fix amino acid hydrazides. Formed precipitates were filtered out, and washed with 2 ml. of water. These treatments were repeated again with 0.2 ml. of isovaleraldehyde. The filtrate and washings were brought together. Remaining traces of hydrazine and amino acid hydrazides were removed by washing the aqueous solution of free amino acid with 5 and 3 ml. of ether. C-terminal amino acids thus obtained were treated with DNFB by the same method as in the case of N-terminal amino acids determination and extracted with 2 per cent sodium bicarbonate and ether. C-terminal-DNP-amino acids were determined by paperchromatographic methods using the above mentioned solvents.

RESULTS AND DISCUSSION

Multiplicity of Conchiolin--The scheme of fractionation of conchiolin by using 70 per cent acetic acid and 2 *N* ammonia as the solvents is presented in Table I. The yield of each fraction is given in Table II.

Table I

The Fractionation Scheme of Conchiolin with 70 per cent Acetic Acid and 2 N Ammonia

Conchiolin, 1.00 g.

70% acetic acid, 100 ml.

Conchiolin I, 600 mg. (soluble, precipitated and washed with acetone, and air-dried)	Conchiolin II, 400 mg (insoluble, washed with acetone and air-dried

2 *N* ammonia, 100 ml.

Conchiolin I-1, 570 mg. (soluble, precipitated and washed with acetone, and air-dried)	Conchiolin I-2, 30 mg. (insoluble, washed with acetone and air-dried)

Table II

The Yield of Fractionated Conchiolin with 70 per cent Acetic Acid and 2 N Ammonia Reagents

Fraction \ Conchiolin	Nacreous layer (%)	Prismatic layer (%)
Conchiolin I	10	60
,, II	90	40
,, I-1	4	57
,, I-2	6	3

In the course of electrodialysis, about 1 per cent of Conchiolin I from the prismatic layer passed through the cellophane membrane and precipitated in the cathodic compartment. This positively charged fraction was not found in conchiolin obtained from the nacreous layer.

The scheme of a fractionation procedure of conchiolin with 14 per cent copper-ethylenediamine solution is quite similar to that of Table III.

Soluble fraction in 14 per cent copper-ethylenediamine solution was named Conchiolin V and insoluble one was named Conchiolin VI.

The yield of Conchiolin III and V are givin in Table IV.

TABLE III

The Fractionation Scheme of Conchiolin with 30 per cent $[Co(py)_4]\ Cl_2$ *Solution*

Conchiolin, 1.00 g.

30% $[Co(py)_4]\ Cl_2$ solution, 100 ml. Centrifugation

Upper layer	Ppt.
Dialysed in running water, 48 hrs., with cellophane membrane, centrifuged down, washed with acetone, centrifuged and air-dried.	Dialysed in running water, 48 hrs., with cellophane membrane, centrifuged down, washed with acetone, centrifuged and air-dried.
Conchiolin III	Conchiolin IV

TABLE IV

The Yield of Soluble Fraction of Conchiolin by the Complex Salt Solutions

Complex salt solution	Conchiolin III 20% $[Co(py)_4]\ Cl_2$		Conchiolin V 14% Copper-ethylene-diamine	
Source / Reaction time	Nacreous layer (%)	Prismatic layer (%)	Nacreous layer (%)	Prismatic layer (%)
3 min.	8	4	64	36
22 hrs.	10	6	80	38

By fractionation, conchiolin from the nacreous layer proved to be more soluble than the scleroprotein from prismatic layer. On the other hand the latter is more soluble in 70 per cent acetic acid and 2 *N* ammonia than the former.

Determination of N-Terminal Amino Acids of Conchiolin—Free N-terminal amino acids of conchiolin were determined by the above mentioned method and the results are summarized in Table V. From these results it is found that every fraction of conchiolin has aspartic acid, glycine and serine as N-terminal amino acids, though complete separation of DNP-glycine and DNP-serine was not successful by this solvent. Better results were obtained by using different developing solvents as shown in Table VI.

With the fractionation of 70 per cent acetic acid and 2 *N* ammonia, no splitting of peptide bonds seems to be occurred, for by this fractionating treatment no new N-terminal amino acid appeared. Splitting of peptide

bond, however, was observed during the treatment of conchiolin from the

TABLE V

N-Terminal Amino Acids of Conchiolin

Solvent: *tert*-Amyl alcohol buffered with equal volume of phthalate buffer (pH 6.0)

		DNP-amino acid or DNP-peptide	R_f	Conchiolin	I	I-1	III[1]	V[1]
Nacreous layer	Acid layer	Peptide	0.00	+	++	++	+	+
		,,	0.10	+	++	++	−	−
		,,	0.23	−	+++	−	+	+
		ε-Lysine	0.28	++++	++++	++++	++++	++++
		Arginine	0.33	+	−	−	−	−
	Ether layer	Aspartic acid	0.04	+++	++	+	+++	+++
		Serine, glycine	0.20	++++	++++	++++	++++	++++
		Proline	0.25	+++	−	−	−	−
		Alanine	0.45	−	−	−	−	−
Prismatic layer	Acid layer	Peptide	0.00	+	+	++	+	+
		,,	0.10	++	+	+++	−	+
		,,	0.23	−	−	−	−	++
		ε-Lysine	0.28	++++	++++	++++	++++	++++
		Arginine	0.33	+++	−	−	−	−
	Ether layer	Aspartic acid	0.04	+++	+	+	+++	+++
		Serine, glycine	0.20	++++	++++	++++	++++	++++
		Proline	0.25	+	−	−	−	++
		Alanine	0.45	−	−	−	−	+++

Number of marks + shows the intensity of yellow color of spots

1) Conchiolin was treated with complex salt for 22 hours

TABLE VI

N-Terminal Amino Acids of Conchiolin I-1 (from the nacreous layer)

Solvent: 10 per cent Ethanol-benzyl alcohol buffered with equal volume of phthalate buffer (M/10, pH 6.0)

	DNP-amino acid	R_f value	Color intensity of spot
Ether extract	Aspartic acid	0.03	+
	Serine	0.18	+ +
	Glycine	0.26	+ + + + +

prismatic layer with copper-ethylenediamine complex salt solution, for alanine

was proved to be added to above three N-terminal amino acids by this fractionation.

Free C-Terminal Amino Acids of Conchiolin—Free C-terminal amino acids of Conchiolin I-1 are given in Table VII. C-terminal amino acids of Conchiolin I proved to be glycine, serine and threonine.

TABLE VII

The C-Terminal Amino Acids of Conchiolin I-1
(from the nacreous layer)

Solvent 1: *tert*-Amyl alcohol buffered with equal volume of phthalate buffer (pH 6.0)

Solvent 2: 10 per cent Ethanol-benzyl alcohol buffered with equal volume of phthalate buffer (pH 6.0)

	DNP-Serine	DNP-Glycine	DNP-Threonine	Dinitrophenol
Solvent 1	+ + + + +		+	+ + +
R_f value	0.22		0.36	0.39
Solvent 2	+	+ + + + +		+ + +
R_f value	0.18	0.28		0.45
Result	+	+ + + + +	+	+ + +

It has been reported that DNP-glycine was less stable against acid hydrolysis than DNP-serine and DNP-threonine (*10*). As shown in Table VII, a large amount of DNP-glycine was detected even after the acid hydrolysis, while DNP-serine and DNP-threonine were found to be present in small amounts. From these facts it is concluded that the most predominant C-terminal amino acid is glycine.

SUMMARY

Conchiolin, a scleroprotein obtained from the shell of the pearl oyster (*Pinctada martensii*), was fractionated with 70 per cent acetic acid, 2 *N* ammonia, the concentrated solution of copper-ethylenediamine, and $[Co(py)_4]Cl_2$ in water, respectively. By these treatments, several fractions of conchiolin were obtained.

The N-terminal amino acids of these fractions of conchiolin were proved to be glycine, serine and aspartic acid. The C-terminal amino acids of Conchiolin I-1 (70 per cent acetic acid and 2 *N* ammonia soluble conchiolin) were glycine, serine and threonine.

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INTESTINAL ABSORPTION OF AMINO ACIDS

I. THE EFFECT OF VITAMIN B_6 ON THE ABSORPTION OF L-AMINO ACIDS THROUGH THE INTESTINE

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Attempts have been made by several workers to elucidate the mechanism of amino acid transport through the cell membrane of animal tissues. In particular the experiments on the absorption mechanism of amino acids through the intestine have provided us with many interesting results. After the original work of Hoeber and Hoeber (*1*) in 1937 and of Schoefield and Lewis (*2*) in 1949, Wiseman (*3*, *4*) in 1955 and 1956 inferred, by using the everted sac method, that L-amino acids were able to pass through the membrane of the rat intestine not only by free diffusion but also by some active principle against the concentration gradient. This was not so in the case of D-amino acids which were shown to be transported only by diffusion. On the other hand, Agar *et al.* (*5*) in 1956 used small pieces of segmented intestine for amino acid uptake and observed that 2,4-dinitrophenol acted so as to diminish the concentration of L-amino acids in the tissues, but with D-amino acids it had no effect.

From these investigations, it is conceivable that some active mechanisms might be concerned in the absorption of amino acids, but their nature is still obscure.

In 1953, Christensen *et al.* (*6*) showed that pyridoxal had an effect on the concentration of amino acids in ascites tumor cells. But Wiseman (*7*) obtained rather contradictory results on the effect of pyridoxal when he studied the active transportation of amino acids with everted intestines *in vitro*. On the other hand, Fridhandler and Quastel (*8*) in 1955 used isolated intestine of guinea pig and observed the inhibitory effect of deoxypyridoxine on L-amino acid transfer, and they also observed that this effect of deoxypyridoxine was not reversed by pyridoxal and pyridoxal phosphate. In 1958 Jacob *et al.* (*9*) showed by the *in situ* technique that the rates of absorption of both L and D-methionine were reduced by the addition of deoxypyridoxine.

In this report, we describe experiments on intestinal absorption by rat intestine both *in vivo* and *in vitro* and provide evidence that B_6 is playing a role in the transfer of L-amino acids during the active absorption.

EXPERIMENTALS

The animals used were young male albino rats weighing 120–150 g., and they were fasted for about 30 hours and then anaesthetized with ether before the experiment. The section of the gut from the jejunum to the ileocaecal region was divided into two equal lengths and placed in the circulation apparatus (Fig. 1). Krebs-Ringer bicarbonate

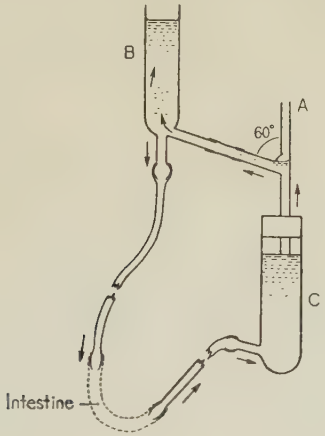

FIG. 1. Circulation apparatus for *in vivo* experiments.
About 20 ml./minute of air was introduced continuously through tube A (diameter 0.4 mm.) into B (1.5×12 cm.) containing the circulating fluid. This caused the fluid to circulate through the intestine and tube C (1.5×9 cm.). There were about 40 ml. of circulating fluid. For details of experiment see text.

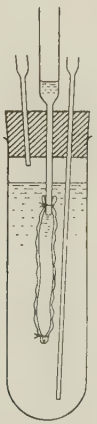

FIG. 2. Wilson's apparatus for the *in vitro* experiments.
At the start of the experiments, the concentration of L or D-methionine on the both sides was brought to 5×10^{-3} *M*. S^{35} DL-methionine was added at a concentration of 20,000 c.p.m./ml. to the external fluid. The reaction tube was preincubated for 10 minutes and then 0.04 or 0.05 ml. samples were taken out at various times. The assay method is shown in text.

buffer containing 0.2 per cent glucose and the amino acids under study was circulated through the intestinal canal. Before irrigation with the test solution, the intestine was washed with 200 ml. of Krebs-Ringer. During the experiment the incubation fluid was kept at 38° and the exposed intestine was covered with moist gauze. The rate of disappearance of amino acids was measured. With this method, we confirmed that the absorption rate of 10^{-2} *M* L-histidine continued steadily for about two hours. In the case of *in vitro* experiments, we used the apparatus reported by Wilson (*10*). It is shown in (Fig. 2). Histidine was measured by the method of Macpherson (*11*), methionine by Bolling's method (*12*) and lysine by Kofranyi's method (*13*). When we used S^{35} DL-methionine, the samples were dried and measured with a Geiger counter (end window type) at infinite thinness.

RESULTS AND DISCUSSION

Active Mechanism of L-*Amino Acid Transportation*—Using Wilson's method and S^{35} DL-methionine, we tested the rate of amino acid transportation from the mucosa to the serosa and *vice versa*. At the start of these experiments, the concentration of both L- and D-methionine on each side of the intestine was equalized by the addition of non-radioactive L- or D-methionine respectively. The initial concentration of these isomers was 5×10^{-3} *M*, and S^{35} DL-methionine was added to the external fluid at the start of the experiment at a concentration of 20,000 c.p.m./ml. When the everted sac was used we could observe the transfer of amino acids from the mucosa to the serosa, as shown in (Fig. 3-A and C).

If the transfer mechanism for each isomer were the same, the amounts of isotope inside the sac would be almost equal whether the experiments were carried out in the presence of the L or D carrier. However, it is difficult to reconcile this with the results shown in (Fig. 3-A and C). It would appear, therefore, that the transfer mechanisms for L- and D-isomers are different. Whereas S^{35} D-methionine was diluted to a negligible extent in the case shown in (Fig. 3-A), the rate of appearance of isotope inside the sac was much faster than that shown in (Fig. 3-C), in which S^{35} L-methionine was diluted by the addition of the L carrier. These results might well be interpreted by considering that the L-isomer is actively transported while transfer of the D-isomer is by simple diffusion. As will be shown later, this conclusion was proved to be correct by *in vivo* experiments.

For results shown in (Fig. 3-B) the inverted sac was used, so the rate of transfer from the serosa to the mucosa was measured. In this case, in contrast with the result shown in (A), the rate of transfer of isotope was markedly decreased. This would readily explained by assuming that from serosa to mucosa the transfer of isotopes is probably by free diffusion.

We could confirm also the findings reported by several workers that 2,4-dinitrophenol (10^{-4} *M*) suppressed the active absorption of L-amino acids to the rate of D-amino acids both *in vivo* and *in vitro*.

Effect of B_6 *on the Absorption of Amino Acids*—Utilizing the anti-B_6 action of L-penicillamine reported by du Vigneaud *et al.* (*14*), 10 mg. of L-penicillamine per 100 g. body weight was injected twice with about 10 hours

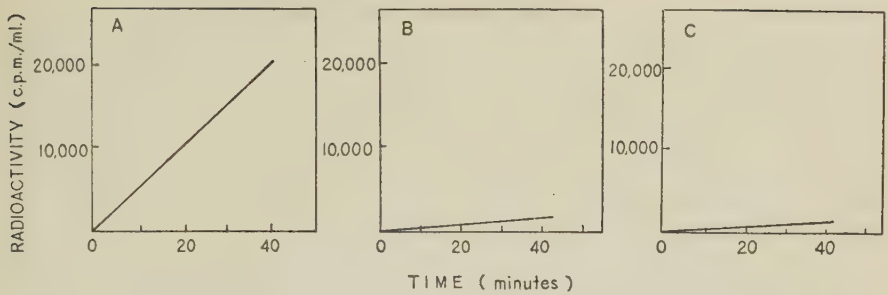

FIG. 3. The rate of transfer of S^{35} DL-methionine. The experimental conditions are given in Fig. 2. and the text. (A) Transfer of S^{35} DL-methionine + D-methionine (5×10^{-3} *M*) from mucosa to serosa. (B) Transfer of S^{35} DL-methionine + D-methionine (5×10^{-3} *M*) from serosa to mucosa. (C) Transfer of S^{35} DL-methionine + L-methionine (5×10^{-3} *M*) from mucosa to serosa.

interval into albino rats. Twenty hours after the second injection, the animals were used for the *in vivo* experiments, as described above. During the circulation of the amino acid solution through the lumen, 500 μg. of pyridoxine was administered through the caudal vein at the time indicated by the arrow in (Fig. 4), and disappearance of amino acids from the circulation fluid was measured with time. The effect of B_6 on L-histidine absorption is shown in (Fig. 4-A).

As may be seen, after injection of B_6, the rate of disappearance was markedly accelerated. In the control experiment, the animal was pretreated with 2 mg. of B_6 instead of L-penicillamine and then tested under the same conditions. (Fig. 4-B) is in clear contrast to (A). The rate of L-histidine absorption in (B) is much faster than that in (A) and the rate is not increased after the injection of B_6. A similar observation was made with B_6 deficient animals by using lysine and methionine. In these case, the L- and D-isomers were tested separately. As seen in (Fig. 5-A), and (B), the rate of absorption of L-isomers was accelerated in the same manner after the administration of B_6. However, no acceleration was observable with D-isomers.

From these experiments it is concluded that in B_6 deficiency the rate of absorption of L-isomers is decreased to that of D-isomers and is restored by B_6 to the normal rate. The ineffectiveness of B_6 towards D-isomers is associated with the fact that D-isomers are taken up only by a simple diffusion. There is a possibility that the effect of B_6 in the above experiments might be caused by the reactivation of transaminase in the intestinal tissue. But the above results seem to exclude this possibility, since neither L-lysine nor L-histidine transaminase have been found in animal tissues.

We observed the absorption of glucose under similar conditions and found there was no effect of B_6. Furthermore, the injection of B_1 or B_2 in B_6 deficiency induced by L-penicillamine had no influence on L-amino acid absorption.

In addition, the rat, which was fed a B_1 deficient diet for a week and

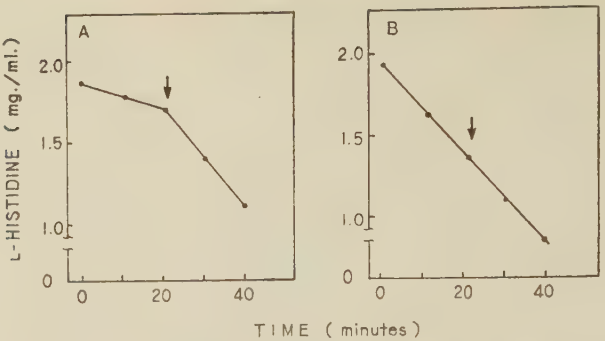

FIG. 4. The rate of absorption of L-histidine from the intestine in B_6 deficient and normal rats.

(A) L-penicillamine treated rat.

(B) B_6 treated rat.

In both cases, 500 μg. of B_6 was injected into the caudal vein at the point indicated by the arrow.

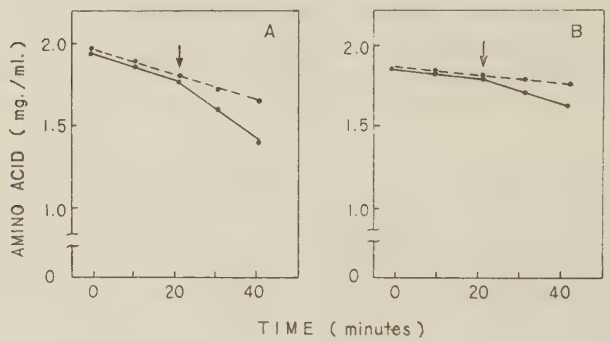

FIG. 5. The rate of absorption of D- and L-isomers of amino acids from the intestine in B_6 deficient rats.

In both cases, 500 μg. of B_6 was injected into the caudal vein at the point indicated by the arrow. Dotted line indicates D-isomer, and solid line L-isomer.

(A) The absorption rate of both D- and L-isomers of methionine.

(B) The absorption rate of both D- and L-isomers of lysine.

showed growth retardation, increase of pyruvic acid concentration in the blood and decrease of B_1 excretion in the urine, was used for the *in vivo* absorption experiment of L and D-methionine, and it was observed that there is no difference of the absorption rate between normal and B_1 deficient rats, and also injection of B_1 did not accelerate it.

It is a problem whether animals pretreated with L-penicillamine are actually in B_6 deficiency. It is well known that xanthurenic acid is excreted in the urine of B_6 deficient rats after the administration of tryptophan. du Vignaud *et al.* (*15*), reported this phenomenon after treatment

of animals with L-penicillamine. We could also confirm this fact in our animals. Normal rats did not excrete xanthurenic acid after the peritoneal injection of 40 mg. L-tryptophan, but 10 hours after the injection of L-penicillamine (15 mg. per 100 g. body weight) injection of the same amount of L-tryptophan resulted in excretion of about 7 mg. of xanthurenic acid during the next ten hour period.

4-pyridoxic acid represents the major end product of B_6 excreted in the urine. du Vignaud *et al.* (*16*) showed that pyridoxal reacts with L-penicillamine to form a thiazolidine compound *in vitro* and claimed that the anti-B_6 effect of L-penicillamine *in vivo* is due to this reaction. Such being the case, after treatment with L-penicillamine the urine of the rat might be expected to contain a smaller quantity of 4-pyridoxic acid than that of normal urine. Five rats, weighing about 150 g., were fed on the basal diet which contained sufficient B_6 (about 300 μg. per rat). Under these condition, on the average about 33 μg. per rat per day 4-pyridoxic acid was excreted. After the injection of L-penicillamine (10 mg. per 100 g. body weight), the urine during the next 24 hours was shown to contain less than 10 μg. of 4-pyridoxic acid per rat. The details of this experiment will be reported in the near future. Through these experiments, we could confirm B_6 deficiency induced by L-penicillamine in our experimental animals which were used for the absorption of amino acids.

SUMMARY

1. Using the *in vitro* method and S^{35} DL-methionine, we tested the rate of amino acid transfer from the mucosa to the serosa of rat intestine and *vice versa*. The transfer rate of the L-isomer from mucosa to serosa was shown to be much faster than that of the D-isomer. However, when the same experiments were carried out from serosa to mucosa, there was no difference between the L- and D-isomer. In this case, the rate of transfer was reduced to that of free diffusion.

2. In B_6 deficiency induced by L-penicillamine *in vivo*, the rate of absorption of the L-isomer is decreased and restored by B_6 to the normal rate. No acceleration of the rate of absorption was observable with the D-isomer. The effect of B_6 was not duplicated by other vitamins such as B_1 or B_2. The absorption of glucose under the same experimental conditions was not influenced by B_6. Furthermore, the rate of L-methionine absorption was not affected in B_1 deficiency.

3. Evidence is given that the animals after injection of L-penicillamine were in B_6 deficiency.

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INTESTINAL ABSORPTION OF AMINO ACIDS

II. ISOTOPIC STUDIES ON THE AMINO ACIDS ABSORPTION FROM THE INTESTINE USING S^{35} DL-METHIONINE AND C^{14} DL-VALINE

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From our previous work (*1*) and reports by many investigators it is generally agreed that the L-isomers of amino acids are actively transported from the lumen of the intestine. With regard to this it is interesting to note the report of Wiseman (*2*) in 1956 that there is rapid transamination between L-glutamic and other keto acids during intestinal absorption. However, as we reported previously, without the participation of transaminase some L-isomers like lysine may be transferred through the cell membrane.

In this paper we deal with the problem of whether the amino acids absorbed are metabolized during absorption.

EXPERIMENTALS

The animals used were young male albino rats, weighing about 150 g. and they were fasted for 24 hours before the experiment. They were anaesthetized with ether. The section of the gut from the jejunum to the ileocaecal region was irrigated with about 200 ml. of Krebs-Ringer bicarbonate buffer at 37°. After drainage, two sections of the gut of about 5 cm. length were ligated without removing their blood circulation. Into one was introduced one ml. of Krebs-Ringer bicarbonate buffer and into the other a solution of the labeled amino acid (total c.p.m. 2×10^5) in the same buffer. Ten minutes later, the animals were desanguinated. Two sections of gut were removed and the incubation fluid thoroughly washed out. The isolated sections of intestine were treated separately as shown in Scheme 1.

For the determination of amino acids which appeared in the portal circulation, the whole small intestine containing the labeled amino acid was ligated, and then the portal blood was sucked up by syringe. The sample was treated with trichloroacetic acid (TCA) and the supernatant was used for the estimation of amino acids.

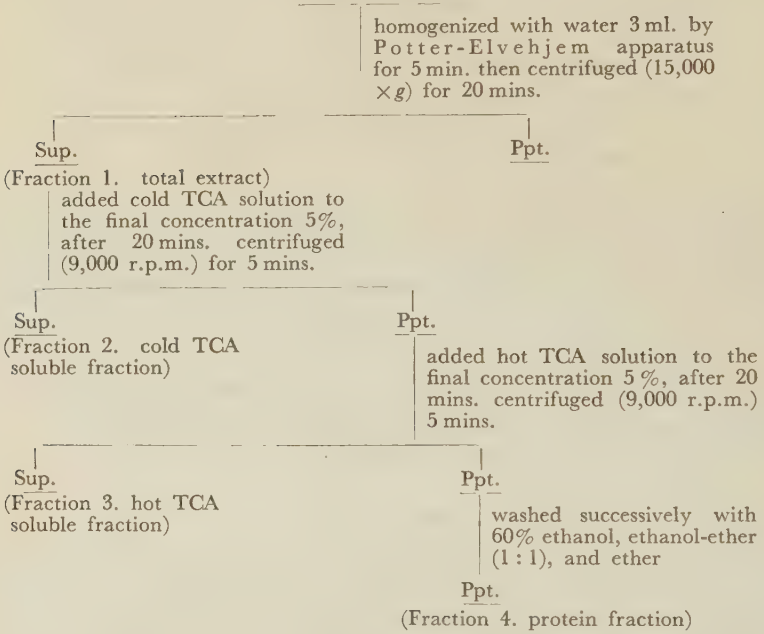
In *in vitro* experiments using the everted intestine, Wilson's apparatus was employed as described previously (*1*). Protein was determined by the improved method of Lowry (*3*). Paper chromatography of amino acids was carried out as follows: The samples were deproteinized with TCA. After removal of the acid with ether, the solution was applied on paper strips. The developing systems were *n*-butanol : 17 *N* ammonia : methylethylketone : water (50 : 10 : 30 : 10 : solvent 1), or secondary butanol : formate : water : (75 : 15 : 10 : solvent 2). Ionophoresis; samples were eluted with distilled water from the spot of the paper strip, about 0.05 ml. of the sample was applied on the paper, the ionophoresis was carried out in phosphate buffer at pH 8.0 (ionic strength 0.1) with

2 m.A. for about 12 hours in the cold room at 4°.

Ascending chromatography was carried out for 12 hours at room temperature. For estimating the radioactivity, the samples were dried and measured with a Geiger counter (end-window type) at infinite thinness or the paper chromatogram was put in a

SCHEME 1

TCA Fractionation



gasflow counter and a rate meter was used to measure the radioactivity. The ratio of total activity of each ninhydrin positive substance could then be estimated.

RESULTS AND DISCUSSION

As mentioned above, the intestinal tissues were fractionated according to (Scheme 1), after incubating with S^{35} DL-methionine for 10 minutes. (Table I) shows results compared with the control, in which there were no labeled amino acids and only Krebs-Ringer bicarbonate buffer.

The cold TCA soluble fraction shows about 8 times as much radioactivity as that of the control. On the contrary the radioactivity of the protein fractions of both control and experimental were almost the same. This finding is also supported by use of C^{14} DL-valine (carboxyl labeled) instead of S^{35} DL-methionine, as may be seen in (Table II).

In the hot TCA soluble fraction, there is no radioactivity of the nucleic acids and their conjugates. Therefore, in our experimental conditions, the amount of amino acid bound to nucleic acids was shown to be negligible. When the incubation time was lengthened to 40 minutes, the situation is not changed.

From these facts it is conceivable that the amino acids absorbed from the intestine may exist in the intestinal wall quite independently of the

TABLE I

The in vivo Absorption Experiment of S^{35} DL-*Methionine*

	Fraction	Total c.p.m.	c.p.m./mg. protein
Experiment[a]	1. Total extract	3,880	
	2. Cold TCA soluble fraction	3,830	
	3. Hot TCA soluble fraction	0	
	4. Protein fraction	32	6
Control[b]	1. Total extract	470	
	2. Cold TCA soluble fraction	420	
	3. Hot TCA soluble fraction	0	
	4. Protein fraction	44	5

a) The intestinal wall incubated with S^{35} DL-methionine.
b) The intestinal wall incubated with Krebs-Ringer.

TABLE II

The in vivo Absorption Experiment of C^{14} DL-*Valine*

	Fraction	Total c.p.m.	c.p.m./mg. protein
Experiment[a]	1. Total extract	4,820	
	2. Cold TCA soluble fraction	4,430	
	3. Hot TCA soluble fraction	0	
	4. Protein fraction	40	10
Control[b]	1. Total extract	107	
	2. Cold TCA soluble fraction	88	
	3. Hot TCA soluble fraction	0	
	4. Protein fraction	35	4

a) The intestinal wall incubated with C^{14} DL-valine.
b) The intestinal wall incubated with Krebs-Ringer.

amino acids which are present in the tissue as the amino acid pool.

The radioactive compounds in the TCA soluble fraction were then analyzed as described above. The paper chromatogram after absorption of S^{35} DL-methionine is shown in Fig. 1. There are two peaks having high radioactivity. The one having the high R_f coincides well with an authentic sample of DL-methionine. To identify the other radioactive peak of low R_f, the substance was collected by repeated chromatography and ionophoresis. The purified compound was dissolved in 3 N HCl and boiled at 120° in a sealed glass tube for 8 hours. After this treatment, the compound was run again on a paper chromatogram as above and it was found that the unknown substance

had been completely converted to methionine by this procedure. When we used 3 N H_2SO_4 instead of 3 N HCl, no such conversion was observable. These results are shown in Fig. 2. Consequently, since the unknown compound might be readily reduced to DL-methionine, the substance of low R_f

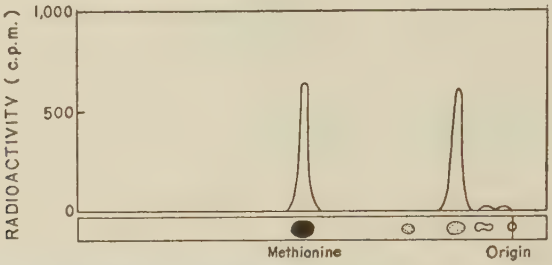


FIG. 1. The paper chromatogram of TCA soluble fraction.

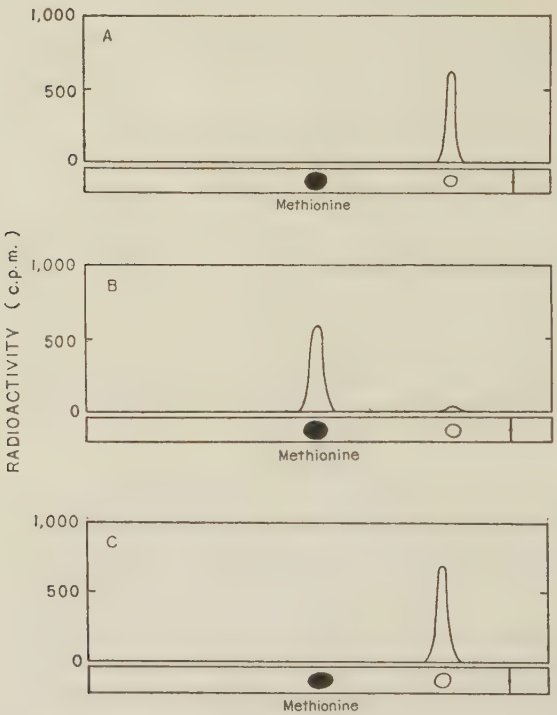


FIG. 2. The paperchromatogram of the low R_f compound under various treatments.

was compared with an authentic sample of DL-methionine-sulfoxide on a paper chromatogram.

As shown in Fig. 3, the unknown compound was identified as methionine-sulfoxide. The above experiments show that about half the DL-methionine

seems to be oxidized to DL-methionine-sulfoxide during absorption. The formation of DL-methionine-sulfoxide was also found *in vitro*, using the everted

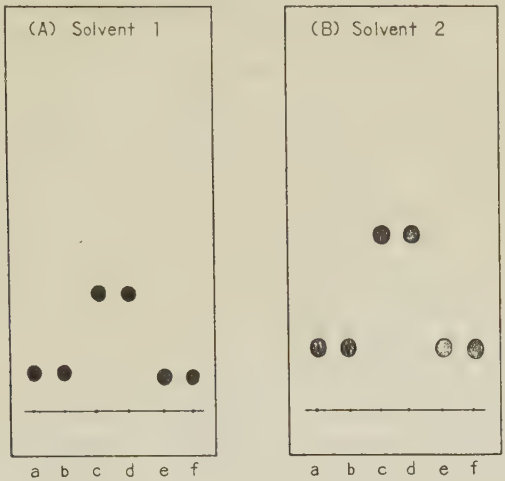


FIG. 3. The paperchromatogram of low R_f compound and methionine-sulfoxide. a: the low R_f substance; b: Meth. S=0; c: the low R_f substance after treatment with HCl; d: Meth. S=0 after treatment with HCl; e: the low R_f substance after treatment with H_2SO_4; f: Meth. S=0 after treatment with H_2SO_4. Meth. S=0 indicates Methionine sulfoxide.

intestine with Wilson's apparatus. At the start of the experiment, 5×10^{-3} *M* DL-methionine was added to both the mucosal and the serosal side and S^{35} DL-methionine (200,000 c.p.m.) was added to the external fluid (the mucosal side). After 30 minutes, the tissue was thoroughly washed, homogenized and treated with TCA. The TCA soluble fraction was chromatographed as

TABLE III

The in vitro Experiment of S^{35} DL-Methionine

1	Intestinal wall	Methionine	212 c.p.m.
		Meth.S=0	40
	Serosal fluid	Methionine	118
		Meth.S=0	2
2	Intestinal wall	Methionine	150
		Meth.S=0	30
	Serosal fluid	Methionine	106
		Meth.S=0	0

Mucosal fluid: 5×10^{-3} *M* DL-methionine+S^{35} DL-methionine.
Serosal fluid: 5×10^{-3} *M* DL-methionine.
Incubated for 30 minutes at 38°.

described above and the corresponding spots were measured by a gasflow counter and a rate meter. As seen in (Table III), about one-fifth of the DL-methionine was converted to its sulfoxide in the intestinal wall, although this amount was not so great as that in *in vivo* experiments. It was interest-

TABLE IV

The in vivo Experiment of S^{35} DL-*Methionine*

	Sample	Methionine : Meth.S=0[a)]
1	Intestinal wall	5 : 4
	Portal blood	50 : 3
2	Intestinal wall	4 : 5
	Portal blood	10 : 1
3	Intestinal wall	1 : 1
	Portal blood	11 : 1

a) Methionine: Meth.S=0 indicated the magnitude of the ratio of total counts in each substance on the paper chromatogram.

ing that in the serosal fluid there is a minute amount of the oxidized methionine. We then analysed the distribution of these two compounds in portal blood after absorption of S^{35} DL-methionine. The experimental conditions were as described above. Five minutes after introducing S^{35} DL-methionine into the intestine, the portal blood was removed and both this blood and

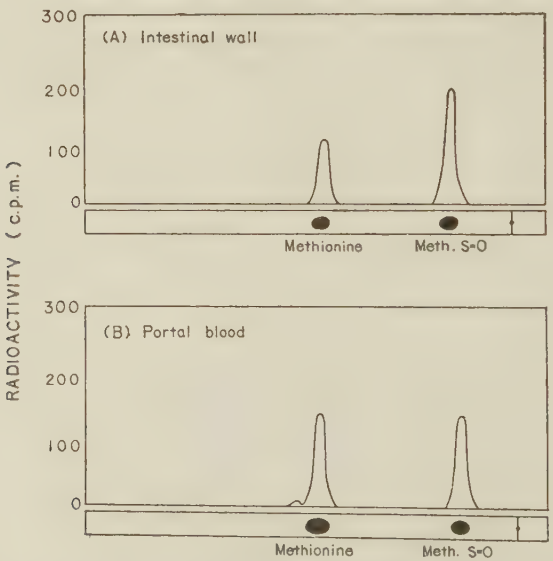


FIG. 4. The paperchromatogram of TCA soluble fraction which was extracted from intestine and portal blood after incubating with S^{35} DL-methionine-sulfoxide.

the intestine were treated as before. After acid treatment the magnitude of the ratio of isotope in methionine and methionine-sulfoxide was estimated in both the supernatant by paper chromatography.

Table IV shows that in portal blood the ratio of methionine to its sulfoxide is much higher than that in the intestinal tissue.

Therefore it is concluded that during absorption methionine is oxidized, and it is again reduced in the portal blood. To confirm this, S^{35} DL-methionine-sulfoxide was prepared and its absorption measured *in vivo* in the same way as S^{35} DL-methionine. As seen in Fig. 4, the magnitude of the ratio of isotopes of these compounds is consistent with the fact that methionine-sulfoxide is subjected to reduction in the intestinal wall or blood system. It is not clear now whether this oxidoreduction of methionine is closely associated with the mechanism of absorption. However, the following evidence is interesting in this connection. Twenty minutes after the injection of S^{35} DL-methionine into the caudal vein, the intestine and the systemic blood were removed, and their radioactivities determined. As seen in Fig. 5, only a minute amount of methionine-sulfoxide was detected in the intestine and the blood. This suggests that, during absorption from intestine, methionine

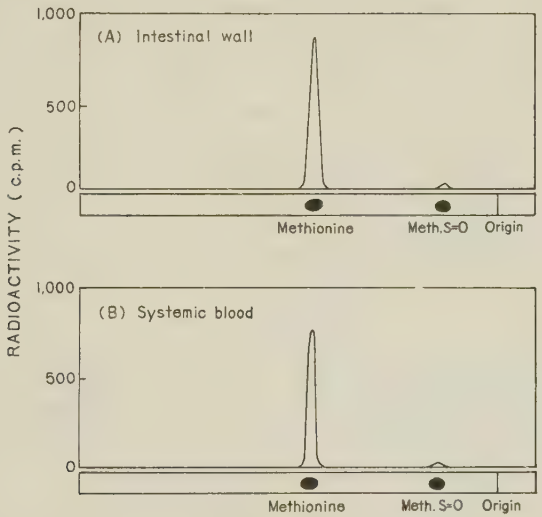


FIG. 5. The paperchromatogram of TCA soluble fraction which was extracted from intestine and systemic blood after injection of S^{35} DL-methionine into the caudal vein.

is more readily oxidized. When C^{14} DL-valine was used instead of methionine, the TCA soluble fraction of the intestine showed only one peak on a paperchromatogram which was identical with DL-valine itself, as shown in Fig. 6. From the above facts, it is conceivable that some amino acids like valine are absorbed unchanged while some like methionine and glutamic acid, as reported by W i s e m a n, are subjected to modification during absorption.

We have no evidence as yet that amino acids might be activated by

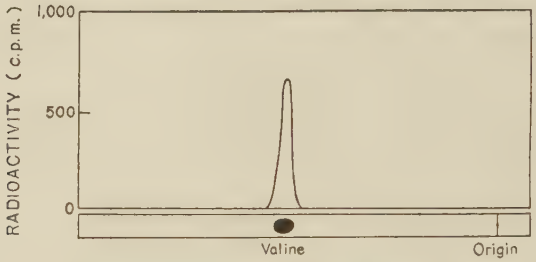


FIG. 6. The paperchromatogram of TCA soluble fraction, which was extracted from intestine incubated with C^{14} DL-valine.

forming adenylate conjugates during absorption, though this will be examined later.

SUMMARY

1. Observation was made on the metabolic pattern of amino acids in the intestinal wall and portal blood after absorption of S^{35} DL-methionine and C^{14} DL-valine.

2. Amino acids after entering the intestinal tissues seem to be independent of the pool of amino acids in the tissues.

3. About half the methionine observed was oxidized to methionine-sulfoxide in the intestine, and was again reduced in the portal circulation.

4. The above oxidoreduction of methionine appears to be coupled with its mechanism of absorption.

5. C^{14} DL-valine enters the portal blood without any modification.

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INTESTINAL ABSORPTION OF AMINO ACIDS

III. INTERFERENCE BETWEEN AMINO ACIDS DURING INTESTINAL ABSORPTION

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The fact that several similar amino acids have a competitive action during absorption from the intestine has been reported by Wiseman (*1*), Ägar *et al.* (2), and Hird *et al.* (3). In 1956, Elvehjem *et al.* (*4*) inferred from nutritional experiments that the inhibition of growth of young rats was caused by the administration of a diet in which the proportions of L-valine, L-leucine and L-isoleucine were unbalanced.

In this paper, we describe the interference between these three amino acids, during absorption from the intestine.

EXPERIMENTALS

The animals used were male rats, weighing about 150 g. Experiments were carried out both *in vivo* and *in vitro*. According to the method described in the previous reports, in *in vivo* studies the rat intestine was divided in two equal lengths and each segment was placed in the circulation apparatus (*5*). One segment was used as a control through which Krebs-Ringer bicarbonate containing the amino acids under study was allowed for circulation. To observe the interference between the amino acids, in the other segment the circulation fluid contained one or two amino acids in addition to those of the control. The disappearance of the amino acid was measured in parallel in the circulating fluid of segments. In *in vitro* experiments, the everted sac method reported by Wilson was employed, as described previously (*5*). The experimental conditions are given later.

Histidine was determined by the method of Macpherson (*6*), methionine by the improved method of McCarthy and Sullivan as reported by Bolling (*7*). Leucine, isoleucine and valine were separated by paperchromatography and determined by a slight modification of Wolfe (*8*) and Kornberg and Patey (*9*). 0.02 ml. of the samples were spotted with deionized water and dried. Chromatograms were developed by the descending technique. The solvent system was *n*-butanol: methylethylketone: 17 *N* ammonia: water (5 : 3 : 1 : 1). After developing for 18 hours at room temperature, the paper strips were dried and warmed on 110–115° for 15 minutes, and the amino acids were located as fluorescent spots in ultraviolet light at 365 mμ. The fluorescent spots and blank areas of paper were cut into strips and each area was eluted for 2 hours with 2 ml. of 0.05

M citrate buffer, pH 5.0, in a test tube. One ml. of each eluate was applied to a column (0.5×5 cm.) of Dowex 50 (Na-form) which had previously been washed by 0.05 *M* citrate buffer, pH 5.0. The columns were washed with one ml. of 0.05 *M* citrate buffer and the total volume of each eluate was measured. One ml. of each eluate was used to determine amino acid by the improved ninhydrin method of Yemm and Cocking (*10*).

RESULTS AND DISCUSSION

Interference between L-*Histidine and* L-*Methionine*—As may be seen in Table I, absorption of L-histidine decreases to about 70 per cent of that of the control in the presence of an equimolar concentration of L-methionine.

TABLE I

Interference between L-*Histidine and* L-*Methionine*

No. of exp.	No. of rats	Concentration of amino acids in circulating fluid (10^{-3} *M*)		Amount of amino acids absorbed (*mg./hr./g. dry weight*)		per cent inhibition
		Histidine	Methionine	Histidine	Methionine	
1	5	5	—	45.3		0
		5	5	32.0		29.4
2	6	5	—	42.8		0
		5	2.5	37.8		11.7
3	5	10	—	78.1		0
		10	2.5	77.9		0
4	5	—	5		33.6	0
		5	5		37.7	0
5	5	—	2.5		17.5	0
		5	2.5		18.9	0
6	6	—	2.5		18.1	0
		10	2.5		17.9	0

In contrast with this, absorption of L-methionine was not affected by the addition of L-histidine. Therefore, the interference of L-methionine with L-histidine is not reciprocal. This is in agreement with results of Wiseman that an amino acid such as L-methionine, absorbed slowly, inhibits the absorption of rapidly absorbed amino acids like L-histidine (*1*). In the above system, when the molar concentration of L-methionine was lowered to one fourth of that of L-histidine, no interference was observable.

Reciprocal Interference between L-*Valine*, L-*Leucine and* L-*Isoleucine*—The first experiments were carried out in *in vivo* as described above. The rates of disappearance of the amino acids under study were measured during 40 minutes.

As shown in Table II, the absorption of L-valine and L-leucine was inhibited by about 50 per cent in the presence of the antagonizing amino acids. In this case, the interference was reciprocal.

Moreover, when both L-leucine and L-isoleucine were present, the inhibition of L-valine was shown to be additive, as may be seen in Table III.

To confirm these results in *in vitro*, the everted intestine was used in Wilson's apparatus. At the start of the experiment, 5×10^{-3} *M* of both DL-valine and D-lysine were added to both the mucosal and the serosal sides

of the intestine to equalize the total concentration of amino acids. Then C^{14} DL-valine (200,000 c.p.m., carboxyl labeled) was added to 10 ml. of the external fluid (the mucosal side). After 20 minutes, 0.1 ml. of the internal fluid was removed. It was dried on a planchet at infinite thinness and the radioactivity measured with a Geiger counter (end-window type). We could therefore mea-

TABLE II

Interference between L-*Valine and* L-*Leucine*

No. of exp.	No. of rat.	Concentration of amino acid in circulating fluid (10^{-3} *M*)		Amounts of amino acid absorbed (*mg./40 min./ g. of dry weight*)		per cent inhibition
		Valine	Leucine	Valine	Leucine	
1	4	10	—	32.4		0
		10	15	13.1		60.0
2	4	—	15		58.7	0
		10	15		29.3	50.1

TABLE III

Interference between L-*Valine,* L-*Leucine and* L-*Isoleucine*

No. of exp.	Concentration of amino acids of circulating fluid (10^{-3} *M*)			Amount of L-Valine absorbed (*mg./40 min./g. dry weight*)	per cent inhibition
	Valine	Leucine	Isoleucine		
1	10	—	—	43.6	0
	10	15	10	9.4	78.5
2	10	—	—	63.6	0
	10	15	10	14.4	77.3
3	10	—	—	57.6	0
	10	15	10	13.6	76.4
4	10	—	—	53.4	0
	10	15	10	14.9	72.1

TABLE IV

The Effect of L-*Leucine,* L-*Isoleucine and both of them on the Transport of* C^{14}-*Valine in vitro*

Exp. No.	Amino acid	Amount of C^{14}-valine[1]	per cent inhibition
1	C^{14}-DL-valine + D-lysine	59	0
	C^{14}-DL-valine + L-leucine	32	46
2	C^{14}-DL-valine + D-lysine	69	0
	C^{14}-DL-valine + L-isoleucine	34	51
3	C^{14}-DL-valine + D-lysine	54	0
	C^{14}-DL-valine + L-leucine + L-isoleucine	13	76

1) This was calculated as c.p.m. in one ml. of internal fluid per mg. dry weight of the intestine.

sure the active transfer of L-valine across the intestinal wall, since D-valine is transported very slowly and only by free diffusion.

To observe the interference of other amino acids like L-leucine or L-isoleucine with the absorption of L-valine, the former was added in place of D-lysine, at a concentration of 5×10^{-3} *M*. When 5×10^{-3} *M* of both L-leucine and L-isoleucine were added as competitors, the concentration of D-lysine in the control rose to 10^{-2} *M*.

As shown in Table IV, the administration of competitive amino acids decreases the rate of absorption of L-valine by about 50 per cent and the effect of these competitors was shown to be synergistic.

These results are quite consistent with those of the *in vivo* experiments described above. L-methionine decreased the uptake of L-valine by about 50 per cent, but L-histidine was without effect.

These studies support the view that between L-valine, L-leucine and L-isoleucine there is a mutual competition during absorption from the intestine. This is specific for these three amino acids since the interference between other amino acids such as methionine and histidine is not reciprocal.

These results were also supported by measurement of the concentration of labeled amino acid taken up by the intestinal tissue. 2 g. (wet weight) of intestinal fragments were slit open, cut into segments of about one cm. length and incubated in Krebs-Ringer bicarbonate solution at 38° for 10 minutes. The incubation fluid was contained 5×10^{-3} *M* of both L-leucine and C^{14} DL-valine (300,000 c.p.m.). In control experiments, 5×10^{-3} *M* of D-lysine was introduced in place of the same concentration of L-leucine. After incubation, the tissues were washed thoroughly with cold Krebs-Ringer solution, and homogenized. After centrifugation at 12,000 r.p.m. for 15 minutes the supernatant was dried and the radioactivity was measured. As seen in Table V, in the presence of L-leucine the radioactivities of the samples diminished to about 50 per cent of the control.

TABLE V

The Effect of L-*Leucine on the Uptake of* C^{14} DL-*Valine by the Intestinal Wall in vitro*

Amino acid	Amount of C^{14} DL-valine taken up per c.p.m./g. of wet weight	per cent inhibition
C^{14} DL-valine+D-lysine	7,260	0
C^{14} DL-valine+L-leucine	3,660	49.6

These results together with the above findings support the view that the competition between these amino acids during transportation through the membrane occurs in the earlier stage of absorption. These findings are suggestive to explain the inhibition of growth of young rats caused by the administration of an inadequate balance of L-valine, L-leucine and L-isoleucine.

SUMMARY

1. The interference between L-valine, L-leucine and L-isoleucine during

absorption from the intestine was studied both in *in vivo* and *in vitro*.

2. These three amino acids have a characteristic competitive action on the absorption mechanism. The interference between other amino acids such as methionine and histidine is not reciprocal.

3. Competition may occur in the earlier stage of absorption and be related to the step of active transportation.

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